



HAL
open science

Production of biological hydrogen by dark fermentation of lignocellulosic residues: Links between substrate composition and structure of fermentative microbial communities

Lucile Chatellard

► To cite this version:

Lucile Chatellard. Production of biological hydrogen by dark fermentation of lignocellulosic residues: Links between substrate composition and structure of fermentative microbial communities. Life Sciences [q-bio]. Institut National d'Etudes Supérieures Agronomiques de Montpellier, 2016. English. NNT : . tel-02800561

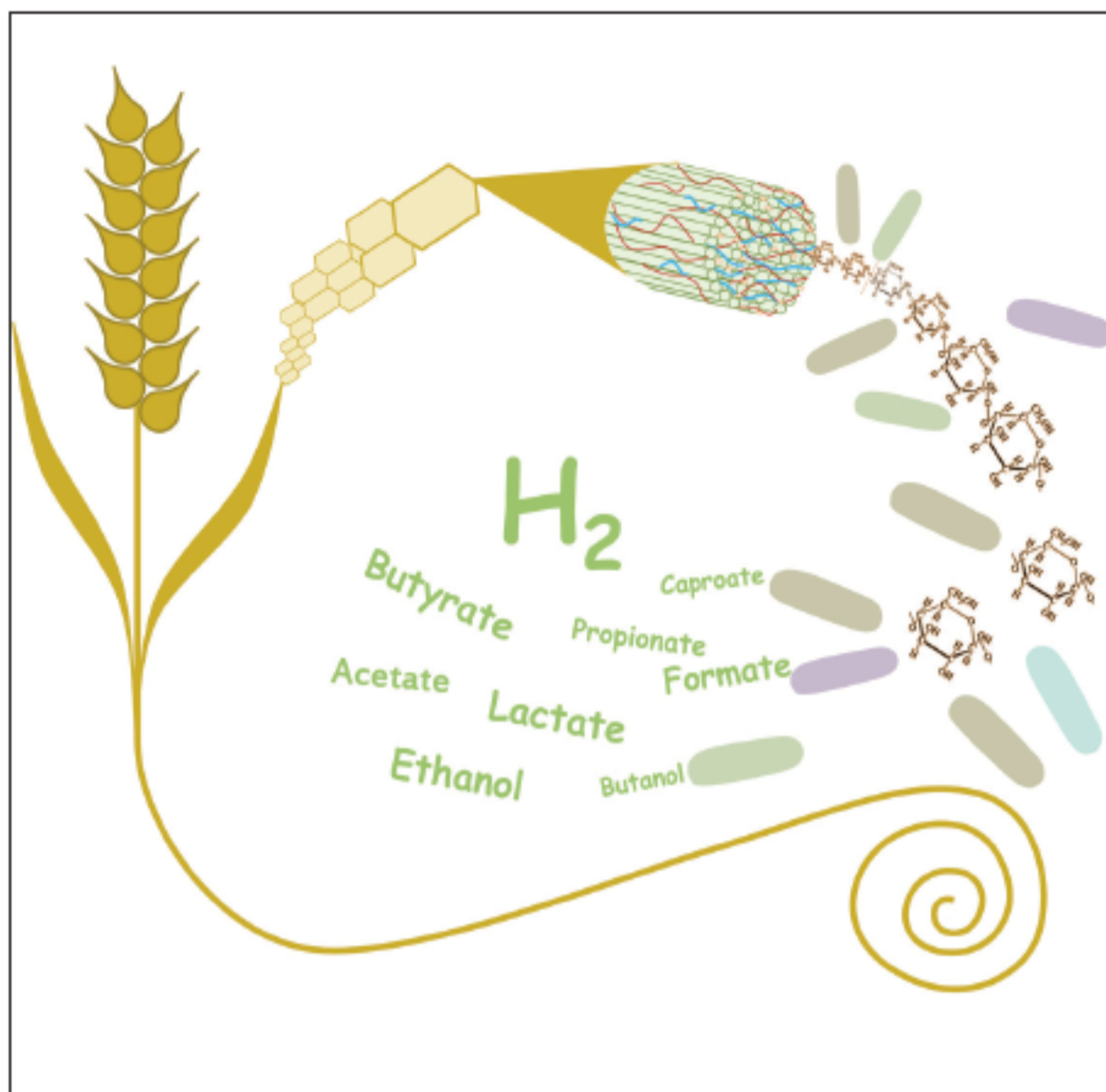
HAL Id: tel-02800561

<https://hal.inrae.fr/tel-02800561v1>

Submitted on 5 Jun 2020

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

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



PRODUCTION DE BIO-HYDROGÈNE PAR FERMENTATION SOMBRE DE RÉSIDUS LIGNOCELLULOSIQUES

LIENS ENTRE STRUCTURE DU SUBSTRAT ET COMMUNAUTÉS
BACTÉRIENNES FERMENTAIRES

*PRODUCTION OF BIOLOGICAL HYDROGEN BY DARK FERMENTATION OF
LIGNOCELLULOSIC RESIDUES:
LINKS BETWEEN SUBSTRATE COMPOSITION AND STRUCTURE OF
FERMENTATIVE MICROBIAL COMMUNITIES*

THÈSE

Pour obtenir le grade de
Docteur

Délivré par Montpellier SupAgro

Préparée au sein de l'école doctorale GAIA
Et de l'unité de recherche Laboratoire de Biotechnologie de
l'Environnement (INRA)

Spécialité : Agrossources, Procédés, Aliments, Bioproduits

Présentée par Lucile CHATELLARD

Production of biological hydrogen by dark
fermentation of lignocellulosic residues:
*Links between substrate composition and structure
of fermentative microbial communities*

Production de bio-hydrogène par fermentation sombre
de résidus lignocellulosiques:

*Liens entre structure du substrat et communautés
bactériennes fermentaires*

Soutenue le 18 Novembre 2016 devant le jury composé de

Mr. Patrick DABERT, DR, Irstea – Rennes

Rapporteur

Mr Pierre FONTANILLE, MdC, Polytech' Clermont-Ferrand

Rapporteur

Mme Carole MOLINA-JOUVE, Pr, LISBP – INRA Toulouse

Examineur

Mr Éric DUBREUCQ, Pr, Montpellier SupAgro

Examineur

Mme Hélène CARRÈRE, DR, LBE – INRA Narbonne

Directrice

Mr Éric TRABLY, IR, LBE – INRA Narbonne

Encadrant

Résumé de la thèse

La fermentation sombre est un procédé biologique naturel qui permet de produire de l'énergie sous forme de dihydrogène (H₂) tout en dégradant des déchets organiques et notamment les résidus agricoles, majoritairement composés de lignocellulose. Ce procédé repose sur l'activité conjointe de microorganismes fermentaires qui, grâce à leur fonctionnement en écosystème, possèdent un potentiel enzymatique performant à la fois pour dégrader des substrats complexes, tels que la biomasse lignocellulosique et produire de l'hydrogène. Cependant, la présence de bactéries non productrices ou consommatrices d'H₂ entraîne des fluctuations de production. Cette thèse a pour objectif d'apporter des éléments de compréhension des mécanismes de sélection des bactéries fermentaires au cours du procédé de fermentation sombre, en relation avec le type de substrat utilisé et les voies métaboliques empruntées. Les expériences menées en réacteurs batch ont permis de mettre en évidence que la structure initiale de la communauté microbienne et la composition du substrat, type de sucre et degré de polymérisation, ont toutes deux une importance capitale dans le déroulement de la fermentation et les quantités d'H₂ produites. Les inocula riches en bactéries du genre *Clostridium* et *Enterobacter* seront plus aptes à produire l'H₂ que ceux dont la proportion en bactéries affiliées aux familles *Bacillaceae* and *Paenibacillaceae* est plus importante. Les résultats démontrent également que sur substrat complexe, des bactéries hydrolytiques de type *Ruminococcaceae* et *Lachnospiraceae* sont préférentiellement sélectionnées. L'utilisation d'un procédé fed-batch sur ce type de substrat a permis d'augmenter les temps de culture et ainsi d'enrichir le milieu en ces bactéries hydrolytiques, même en présence d'une forte abondance relative initiale de bactéries de la famille des *Enterobacteriaceae*. Cette thèse apporte de nouvelles informations sur la dynamique des communautés bactériennes au sein des procédés fermentaires et ouvre ainsi de nouvelles perspectives sur le management des cultures mixtes en vue de produire de l'hydrogène vert et renouvelable.

Mots clefs : Fermentation sombre, Hydrogène, Biomasse lignocellulosique, Sucres, Métabolismes anaérobies

Thesis summary

Dark fermentation is a natural biological process that can be used to degrade organic waste, such as lignocellulosic components, and to produce energy under the form of hydrogen (H₂). The process is based on the activity of fermentative microorganisms in consortia that develop an efficient enzymatic activity to degrade complex substrate as lignocellulose. The presence of hydrogen non-producing or consuming bacteria in the system can result in gas production fluctuation. This thesis aims to highlight the mechanisms behind the selection of fermentative bacteria during dark fermentation, regarding the type of substrate and the final metabolisms. Batch experimentation brought out that both inoculum community structure and substrate composition, i.e. type of carbohydrate and degree of polymerisation, are important for the fermentation progress and thus hydrogen production. Inocula rich in bacteria from *Clostridium* sp. and *Enterobacter* sp. genera appeared to be more likely linked to H₂ production than mixed culture rich in microorganisms affiliated to *Bacillaceae* and *Paenibacillaceae* families. Results also showed hydrolytic bacteria from *Ruminococcaceae* and *Lachnospiraceae* families are preferentially selected on complex substrates. The use of fed-batch process on these types of substrates increased the experimental time and was used to enrich the microbial culture into these bacteria. Overall, this thesis provides new insights on the dynamics of bacterial communities within the dark fermentation process. It opens up new perspectives on mixed culture management to produced green and renewable hydrogen.

Key words : Dark fermentation, Hydrogen, Lignocellulosic biomass, Carbohydrates, Anaerobic metabolisms

« L'architecte du futur construira en imitant la nature, parce que c'est la plus rationnelle, durable et économique des méthodes. »

Antoni Gaudí, 1889

Remerciements

Me voici arrivée au terme d'un chapitre de mon histoire. Bien qu'il représente le point final de cette thèse, il n'en reste pas moins le commencement d'une vie de chercheur qui, je l'espère, continuera à m'apporter autant de bonheur que celui auquel j'ai pu goûter durant ces quelques années au LBE. Mais cette réussite n'aurait pas pu être possible sans l'aide ni le soutien de toutes les personnes qui m'ont entouré pendant ces quatre années au laboratoire, mais aussi depuis le jour où je me suis passionnée pour la Biologie.

C'est pourquoi je souhaite remercier avant tout la personne qui m'a fait confiance pour mener à bien ce projet de thèse, ma directrice de thèse **Hélène CARRERE**. Ton encadrement a tout simplement été exemplaire. Tu m'as à la fois laissé expérimenter ce que je voulais même si ça ne menait pas toujours à grand-chose, mais aussi beaucoup guidé dans l'orientation de la thèse. Merci pour ta patience pendant mes moments de grand stress et ton écoute pendant les coups de mou. Je te fais la promesse que ma prochaine leçon sera d'apprendre à dire NON ! Parce que, en effet, tu ne seras plus là pour me rattraper lorsqu'on me proposera des projets qui ne rentrent pas dans mon emploi du temps... Merci pour m'avoir poussé à te suivre au trampoline (même si finalement je n'y suis pas allée souvent, ça m'aura entraîné à m'inscrire à la GNL et à me remettre au sport, ce qui n'était pas gagné...). De tout cœur merci pour tout !

Bien évidemment je n'oublie pas la deuxième personne qui m'a également suivi pendant ces trois ans, mon encadrant **Eric TRABLY**. Tu as fait bien plus que de m'encadrer puisque que tu m'as toi aussi fait entièrement confiance en me laissant participer à de nombreux projets que ce soit pour la rédaction du chapitre de livre, avec le Lycée Louise Michel ou avec le réseau latino-américain (je n'oublierai pas l'épisode Valentina la veille de son arrivée, tu peux compter là-dessus ^^). Toutes ces activités annexes que tu m'as permis d'accomplir pendant la thèse ont été plus que formatrices. Mais ton encadrement ne s'est pas limité au labo puisque même à l'extérieur tu as été beaucoup présent. Je n'oublierai pas nos sorties course de dernière année entre midi et deux (Allez Lucile !! Plus vite !! On finit par un sprint !! NONNNNNN !!). Pour tous ces moments un grand merci !

Je remercie également **les membres de l'Ecole doctorale GAIA**, filière APAB (ou plutôt les membres de l'ancienne école doctorale SPSA) qui m'ont accordés la bourse ministérielle qui m'a permis de réaliser cette thèse. Un merci tout particulier à **Jean-Philippe DELGENES** pour m'avoir encouragé et fait répéter pour cette épreuve (j'en profite pour te remercier de m'avoir offert l'opportunité de réaliser quelques cours de TP à l'IUT et un MOOC qui pour le coup, je l'espère, ne restera pas graver dans les mémoires de Youtube... Merci beaucoup pour toute ta confiance)

Je remercie les membres du jury de thèse, **Patrick DABERT**, **Pierre FONTANILLE**, **Carole MOLINA-JOUBE** et **Eric DUBREUCQ** pour avoir jugé mon travail et m'avoir délivré ce titre de Docteur. Merci pour toutes vos remarques constructives et pour les riches discussions qui ont découlé de la soutenance. Merci également aux membres de mes comités de thèse, **Théodore BOUCHEZ**, **Frédérique HAM-PICHAVANT** et **Marianne QUEMENEUR**. Votre avis m'aura énormément aidé pour mener à bien cette thèse.

Merci aussi à **Jean-Philippe STEYER** pour m'avoir permis d'entrer dans ce laboratoire pour faire mon stage de Master il y a de cela 4 ans. Nos discussions pendant les repas de Noël resteront toujours gravées dans ma mémoire. Merci beaucoup pour toute ton attention et ton aide pour m'orienter dans cet immense brouillard qu'est le futur.

Un grand merci à **Claire DUMAS** qui finalement aura été m'a première encadrante au laboratoire lors de mon stage **INOUBLIABLE** sur les insectes (les grillons fuyards dévoreurs de moustiquaire n'auront pas eu raison de nous !). Si j'ai réussi à faire cette thèse c'est en grande partie grâce à toi. **MERCI !** Merci aussi à **Jean-Jacques GODON** et de nouveau **Hélène** qui m'ont également encadré sur ce projet qui n'était pas des plus évidents.

Un merci tout particulier à la Dream Team, **Sylvie, Annie, Nadine, Alexandra, Ingrid** et **Véronique**, toujours là quand il y a un pépin dans le grand flou artistique qu'est l'administration. Merci de m'avoir supporté lors de l'organisation du congrès de Montpellier, lorsqu'il fallait organiser les allers et retours de tout le monde, mais aussi pour toutes les fois où j'oubliais de badger en arrivant et avant de partir. Merci pour votre patience, merci d'être là !

J'arrive déjà à deux pages de remerciements et pourtant la liste est encore très longue... La recherche est un travail d'équipe et la force du LBE est que cette équipe représente le laboratoire tout entier. Tous ont eu une grande part de responsabilité dans mes travaux et je vais essayer de n'oublier personne.

Merci à **Kim** pour toute ton aide pour l'analyse des données de séquençage (la publie et en cours de rédaction, je te le promets !). Merci à **Anaïs** pour m'avoir **ENORMEMENT** aidé pour l'extraction et le séquençage des derniers échantillons. Merci à **Roman** d'avoir supporté mes vas-et-viens à son bureau pour tenter de comprendre les stats (ce n'est pas encore gagné mais je m'en approche...). Merci à mes deux stagiaires **Camille** et **Marine** qui ont été plus que compétentes et qui ont permis d'avancer grandement ces travaux de thèse. Merci à **Clément** pour ton aide sur Phyloseq (accroche-toi et ta thèse sera au top !). Merci à **Domi** et **Nadine D.** pour leur soutien, **Eric L.** pour les superbes sorties « Goélands », **Jérôme** et **Marjolaine HML** pour leur aide et leur disponibilité, **Nori** pour nos conversations et nos sorties course, **Fred** pour sa prépa physique (ça va bien se passer !) **Diana** pour m'avoir permis d'organiser le workshop BFFM et d'avoir participé au GdR Symbiose, **Arnaud** pour les cours à SupAgro, à **tous ceux de la HBE** pour leur présence et leur patience lors des TP de L3P, **Philippe** pour son aide précieuse, et **tous les autres** qui auront aussi eu leur part de responsabilité dans mon travail.

Merci à **Elsa** et **Violette** avec qui j'ai commencé mon aventure LBE (notre année la plus mémorable restera la première et nos soirées Echoppe-Salade de choux). Puisque nous sommes sur cette première année, une pensée à tous ceux que j'ai rencontré à ce moment -là, **Mathilde** (et notre mémorable soirée de gala à Saragosse), **Quentin, Elisabeth, Rémi, Maximilien, Mokhles** (bonne chance pour la fin !), **Emilie, Ivan, Caroline, Jean-Charles** (ton expérience aura été plus que constructive), et tous ceux que malheureusement j'oublie... Merci à ceux qui ont été là pendant la thèse, **Cyrille** (on en sera venu à bout !), **Thibaut** (carton rouge !), **Julie** (ma voisine et sœur de chat), **Angel et Gabriel** (Gracias ! mi espanol es mui mejor no ?), **Quentin** (et sa main verte), **Felipe – Maria – Nicolas – Ulysse** (mes co-bureaux de quelques temps), **Wendy Diane et Hélène** (merci d'être ce que vous êtes les filles !), **Amandine** (On achèvera INSYME un jour ou l'autre!), **Antonella** et **toute la team du workshop** (on aura survécu à BFFM !), **Izabel** (bonne chance à toi et ta petite famille), **Géraldine** (sans qui il n'y aurait pas eu de fumier...) mais aussi **tous les autres**, de tout cœur merci !

Pour fermer cette parenthèse labo, je tiens à remercier les trois personnes qui auront le plus compter pendant ces trois ans (tout le monde à son importance, mais sans eux la fin aurait été très difficile...). Merci à **Charlotte**, on ne se sera rencontrée que les derniers mois de ma thèse mais quelle rencontre ! Nos profondes discussions auront été une bouffée d'oxygène et j'espère que ce monde idéal que nous imaginons au plus profond de nous sera un jour réalité :-). Merci à **Flo**, toi et ta joie de vivre, heureusement que vous étiez là ! Nos soirées Musique et nos grandes incompréhensions vont me manquer... Mais ce n'est que partie remise (et oui, tu ne vas pas te débarrasser de mes blagues comme ça, crois-moi! 盲目的駱駝是不是害怕黑暗). Enfin merci à ma **Loulou** ! Quatre ans que nous nous connaissons à présent et heureusement que tu as été là dès le début. J'ai trouvé chez toi une véritable amie, toujours prête à se plier en quatre pour aider tout le monde, peu importe ce qu'on pense de toi. Je sais que la vie au labo n'aura pas toujours été facile et je souhaite de tout cœur que tout le monde se rende compte à quel point ta bonté supasse leur jalousie. Merci d'avoir été et d'être là ! Cette histoire est faite pour durer, j'en suis sûre ;-)

Merci à **mes amis**, mes cinq doigts de la main... Vous m'avez toujours soutenu où que je sois, à La Rochelle, en Ecosse ou à Narbonne. Un retour sur Toulouse et c'est comme s'il ne s'était rien passé, vous êtes toujours avec moi. Nos vies vont prendre des chemins différents à présent (et ça va commencer par toi Vin's avec ton petit à venir :-)) mais j'ai la conviction que peu importe, nous nous retrouverons toujours quelques parts et tout restera inchangé. Merci d'être là, merci pour tout.

Parce que sans eux rien n'aurait été possible, je tiens à remercier toute **ma famille**, qui aura été nombreuse à venir m'écouter pendant ces 2h de soutenance. Merci tout particulièrement à **mes parents** qui m'ont toujours soutenu dans mon choix d'étude même s'ils ne savaient pas trop ce que je faisais ni où ça allait me mener. Ils m'ont permis de faire tout mon cursus comme je l'entendais en me donnant une confiance aveugle sur ces décisions. Même si parfois mon caractère de cochon peut vouloir dire le contraire, je vous en suis énormément reconnaissante. Je vous aime de tout mon cœur et ne vous remercierai jamais assez pour tout ce que vous m'avez donné. Un grand merci à **mes grands-parents** car c'est aussi en grande partie grâce à eux que j'en suis là aujourd'hui. Il y a l'aide financière, certes, mais il y a surtout l'affection que vous me portez chaque jour et que j'espère vous rendre à sa juste valeur. Je sais que partir faire un IUT en Biologie vous a fait peur, surtout à toi papi, mais je sais aussi qu'aujourd'hui nos consciences se sont accordées et que vous voyez l'importance que peut avoir une telle matière dans notre monde et notre vie de tous les jours. Je ne sais pas on arrivera à sauver notre terre un jour, mais vous pouvez être sûrs qu'on se donnera les moyens pour y arriver.

Enfin, merci de tout cœur à ma « nouvelle famille », les **Berger**, pour m'avoir intégré parmi vous et pour être venu également me soutenir le grand jour. Merci surtout à toi mon chéri, **Damien**, pour m'avoir supporté pendant toutes ces années. Je sais que je t'ai donné du fil à retordre, surtout ces derniers mois... mais heureusement que tu étais là car tu m'as fait prendre le recul nécessaire sur mon travail pour ne pas exploser pendant ces trois ans et sans ça je n'y serai pas arrivé de la même façon. Ca y est, Narbonne c'est terminé, nous allons pouvoir profiter de nous et vivre comme nous l'entendons maintenant.

Résumé étendu

Le système industriel sur lequel est basée la société moderne entraîne l'émission d'importantes quantités de gaz à effet de serre qui sont reconnues comme principale cause du dérèglement climatique (Cook et al., 2016). La fabrication intensive de biens pour répondre à la demande toujours croissante de la population a entraîné une forte diminution des ressources naturelles qui, associée à une forte accumulation de déchets, contribue à dégrader l'environnement (Gao and Tian, 2016; Toth and Szigeti, 2016). Pour éviter des scénarios catastrophes depuis longtemps imaginés par les cinéastes, les Hommes doivent dès à présent changer leur mode de vie et envisager de nouvelles manières de produire et de consommer, sans atteintes à l'environnement. Dans cette optique, les gouvernements des pays les plus développés ont récemment signé lors de la COP21, Conference of the parties, les accords de Paris qui visent à mettre en place des actions pour maîtriser la pollution émise par l'activité des Hommes et ainsi ne pas dépasser une hausse de température de 2°C en comparaison aux températures observées au début de l'ère industrielle (UNFCCC. Conference of the Parties (COP), 2015). Parmi ces actions, le traitement des déchets et la transition vers l'utilisation d'une énergie propre et renouvelable apparaissent essentiels pour préserver la Nature.

Contrairement aux énergies dites fossiles, les énergies renouvelables sont produites à partir de ressources naturelles qui sont considérées comme inépuisables à l'échelle humaine, telles que l'énergie solaire, le vent, les marées, la géothermie et la biomasse. De plus, leur mode de production a un plus faible impact environnemental que les énergies fossiles dont l'extraction génère d'importantes quantités de dioxyde de carbone. De nombreux programmes visent à promouvoir la recherche et le développement des énergies renouvelables. A titre d'exemple, 5 931 milliards d'euros ont été débloqués par l'Union Européenne pour financer de tels projets dans le cadre des programmes Recherche et Innovations Horizon 2020 entre 2014 et 2020 (European Commission, 2016).

Parmi les sources d'énergie pouvant se substituer aux énergies fossiles, la biomasse a un fort potentiel. Elle est utilisée depuis des milliers d'années pour le chauffage et l'éclairage. Son utilisation énergétique a été réorientée depuis le XIXème siècle pour les transports, mais elle reste la principale ressource renouvelable actuellement utilisée. La biomasse est la matière organique qui compose chaque être vivant. Dans le cadre de la production d'énergie renouvelable, la biomasse végétale est principalement utilisée de par sa disponibilité et sa composition en sucres solubles et/ou polymérisés. Par biodégradation par des microorganismes tels que les levures ou les bactéries, ces

sucres vont pouvoir être convertis en molécules à haute valeur énergétique comme l'éthanol ou le dihydrogène, et ainsi être utilisées à des fins énergétiques.

Depuis le début du XX^{ème} siècle, des cultures (e.g. céréales, betteraves, canne à sucre...) ont ainsi été dédiées à la production de bioénergies dites de première génération. Mais l'utilisation de ces cultures pour la production d'énergie alors qu'une personne sur neuf souffre de la faim dans le monde a engendré des problèmes éthiques. De plus, la mise en place de ces monocultures a fortement contribué à la déforestation et au déclin de la biodiversité (Carriquiry et al., 2011). De ce fait, la production de bioénergies s'est tournée vers l'utilisation de biomasse dite de deuxième génération, soit la biomasse lignocellulosique et les déchets organiques. Cette biomasse concerne notamment la fraction résiduelle non-comestible de la biomasse agricole dont la transformation en énergie peut à la fois répondre à une problématique de gestion des déchets organiques et à celle de la production de bioénergies.

Les résidus agricoles sont principalement composés de lignocellulose dont la structure complexe et hétérogène contient plus de 66% de sucre sous forme polymérisée. Sa conversion par voie biologique nécessite une première étape d'hydrolyse qui va permettre de libérer les sucres simples qui pourront alors être transformés en bioénergie de la même manière que les sucres issus des biomasses alimentaires. Comme toute matière organique, la lignocellulose est composée principalement de quatre éléments dont le carbone, l'oxygène, l'azote et l'hydrogène. Ce dernier est le plus petit élément chimique présent sur Terre, qui combiné à un autre atome d'hydrogène forme le dihydrogène (appelé plus communément hydrogène, H₂), un gaz dont la valeur énergétique est supérieure à la majorité des vecteurs énergétiques actuellement utilisés. De plus, sa combustion ne génère que de l'eau, faisant de lui un vecteur énergétique propre et un sérieux candidat pour remplacer les énergies fossiles telles que le pétrole.

L'hydrogène peut être produit par un procédé biologique appelé la fermentation dite sombre, par opposition à d'autres systèmes biologiques qui nécessitent de l'énergie lumineuse pour fonctionner. Le procédé est basé sur l'activité anaérobie de certains microorganismes qui sont capables de dégrader et convertir la matière organique en hydrogène. Ces microorganismes colonisent des milieux naturels et anthropiques tels que les marais, les sédiments, les fumiers ou encore les boues de stations d'épuration, et sont de ce fait facilement accessibles.

L'utilisation de la fermentation sombre pour la production de l'hydrogène par conversion de sucres simples a prouvé le potentiel de cette méthode. Ainsi, un maximum de 2,5 moles d'H₂ peut

être produit à partir d'une mole de glucose. En ce qui concerne les substrats complexes comme la lignocellulose, les quantités d'hydrogène sont souvent plus faibles à cause de la première étape d'hydrolyse requise pour libérer les sucres qui pourront ensuite être convertis en H₂. De plus, à cause de l'hétérogénéité de la matière, la dégradation de la lignocellulose nécessite un équipement enzymatique complexe composé de différentes enzymes hydrolytiques. Les consortia microbiens cités précédemment ont la capacité de fournir ces fonctions enzymatiques complémentaires et ainsi améliorer la biodégradation de la biomasse (Jobard et al., 2014). De plus, l'avantage de ces cultures mixtes est qu'elles ne nécessitent pas de travailler en conditions stériles et peuvent facilement s'adapter aux modifications des conditions de culture.

Cependant, l'hydrogène produit pendant la conversion de la matière organique par fermentation ne représente que 20% de la Demande Chimique en Oxygène (DCO) qui compose le substrat. Les 80% restants sont transformés en métabolites fermentaires, coproduits pendant la fermentation. De plus, des microorganismes consommateurs ou non-producteurs d'hydrogène peuvent être présents dans les cultures mixtes et réduire considérablement les rendements de production d'H₂.

A cause de la complexité du système, la dynamique des communautés microbiennes en fermentation sombre au regard de la composition du substrat et du métabolisme fermentaire observé reste incomprise. L'émergence de nouvelles techniques d'analyses biomoléculaires a cependant permis l'expansion des études sur la structure des communautés microbiennes en milieu complexe. Ces avancées offrent la possibilité de mieux comprendre les procédés de fermentation et ainsi proposer des solutions pour améliorer la production d'hydrogène.

Cette thèse s'inscrit dans cette démarche, en se focalisant sur la tri-interaction existant entre les consortia bactériens, la composition du substrat et les métabolismes fermentaires. Pour ce faire, ces travaux se sont articulés en trois axes. Dans un premier temps, l'impact de la composition moléculaire du substrat a été étudié en utilisant une culture mixte issue d'un fumier. Ensuite, l'impact de la structure de la communauté initiale sur le métabolisme fermentaire final a été exploré en utilisant cinq cultures fermentaires différentes sur substrat simple, le glucose. Enfin, une stratégie de sélection d'une culture fermentaire capable à la fois d'hydrolyser la matière et de produire de l'hydrogène a été proposée.

La première partie de l'étude s'est donc concentrée sur l'impact de la composition du substrat, composition moléculaire et degré de polymérisation, sur la sélection de la communauté bactérienne et les voies métaboliques associées.

L'expérience a été réalisée en réacteur batch sur sept substrats représentant différentes fractions de la lignocellulose. Le glucose, le cellobiose et la cellulose micro-cristalline (CMC) sont les hexoses qui ont été choisis pour représenter la cellulose avec différents degrés de polymérisation, tandis que des pentoses, l'arabinose, le xylose et son polymère le xylane, ont été sélectionnés comme composants majoritaires des hémicelluloses. Enfin, la paille de blé a été utilisée comme résidu lignocellulosique modèle.

Dans un premier temps, une culture mixte, ou inoculum, fraîchement extraite d'un digestat de fumier a été utilisée pour réaliser la fermentation. Pour la fermentation des monomères, les résultats ont montré que les quantités finales d'hydrogène produites étaient différentes selon la composition moléculaire du sucre utilisé, hexose ou pentoses. De faibles productions d'hydrogène ont été obtenues sur glucose, en lien avec une production de lactate, métabolite fermentaire dont la production empêche l'accumulation d'hydrogène dans le milieu. Au contraire, trois fois plus d'hydrogène a été produit lors de la fermentation des pentoses au cours de laquelle un métabolisme producteur de butyrate a été observé. Cette différence de métabolisme a pu s'expliquer par la sélection de deux communautés bactériennes différentes au cours de la fermentation. En effet, la communauté bactérienne développée sur hexose était composée majoritairement de bactéries affiliées aux *Paenibacillus sp.* et aux *Bacillus sp.*, bactéries productrices de lactate, alors que des bactéries du type *Clostridium sp.*, réputées pour produire de l'hydrogène, ont majoritairement été sélectionnées sur pentoses.

En ce qui concerne les polymères de sucres, CMC, xylane et paille, de faibles quantités d'hydrogène ont été obtenues, dues au faible taux de dégradation de la matière lorsque la production d'hydrogène était maximale. Ce phénomène a été associé au besoin d'hydrolyser la matière avant conversion des sucres en hydrogène, ce qui limite la production de ce gaz. Néanmoins, l'analyse des communautés bactériennes développées sur ces substrats complexes a montré la sélection majoritaire de bactéries hydrolytiques de la famille des *Ruminococcaceae*.

L'utilisation de ce même inoculum mais après avoir été soumis à un stockage de deux mois à 35°C sans alimentation a conduit à des conclusions différentes, démontrant le fort impact de la culture initiale sur le procédé de fermentation. Contrairement à l'expérience précédente, les mêmes

profils métaboliques ont été identifiés sur les sucres simples, indépendamment de leur composition moléculaire, hexose ou pentose. De faibles productions d'hydrogène ont été obtenues, 15% de la quantité maximale théorique, associées à une production majoritaire d'acétate. Le développement d'une activité homoacétogène, consommatrice d'hydrogène, a ainsi été suspecté, résultant du stress provoqué par le jeûne. Ce métabolisme commun a été le résultat de la sélection d'une communauté bactérienne similaire dans le cas de tous les sucres simples. Cette communauté était composée à la fois de *Bacillaceae* et de *Clostridiaceae*. Dans le cas des substrats complexes, toujours peu d'hydrogène a été produit, mais une augmentation du taux de conversion du xylane et de la paille d'environ 50% par rapport à l'essai avec l'inoculum frais a été observée. Comme dans l'expérience précédente, des bactéries affiliées à la famille *Ruminococcaceae* ont été identifiées en majorité sur ces substrats complexes, avec d'autres bactéries hydrolytiques de la famille des *Lachnospiraceae*.

En comparant les communautés bactériennes sélectionnées à partir des deux inocula, il a pu être observé que lorsque des substrats complexes étaient fermentés, la communauté bactérienne convergait vers une structure commune, permettant une hydrolyse de la matière. Au contraire, la structure finale sélectionnée à partir de sucres simples apparaît dépendre de l'inoculum. Un tel impact de l'inoculum peut cependant avoir de sérieuses conséquences sur le procédé de fermentation, et donc sur la production d'hydrogène.

De ce fait, la deuxième partie de cette thèse s'est penchée sur l'impact de la structure de la communauté initiale sur le procédé de fermentation sombre, soit la sélection de la communauté bactérienne et les métabolismes fermentaires développés. L'expérience s'est focalisée sur la fermentation du glucose en réacteur batch à partir de cinq types de cultures mixtes différentes : digestat de fumiers frais et stocké, boues activées fraîches et stockées en laboratoire ou en station d'épuration. Les résultats ont montré que les boues activées présentaient une meilleure activité de production d'hydrogène que les digestats de fumier. Ainsi, 4 à 5 fois plus d'hydrogène a été produit à partir des boues activées en comparaison aux inocula issus du digestat de fumier. Un métabolisme producteur en majorité de butyrate et d'acétate, idéal à la production d'hydrogène, a pu être observé. Ce métabolisme a été associé à la sélection exclusive de bactéries du genre *Clostridium sp.* Le stockage des boues a eu un effet négatif sur le procédé, conduisant au développement de voies métaboliques concurrentes à la production d'H₂ telles que les voies productrices de propionate ou d'éthanol.

Cependant, les critères d'un inoculum qui affectent la sélection bactérienne et le métabolisme associé sont encore incompris et très peu abordés dans la bibliographie. En raison de leur structure

complexe, les communautés bactériennes qui composent les inocula sont rarement étudiées bien qu'elles pourraient permettre de comprendre le déroulement de la fermentation.

Lors de cette étude, il a été démontré que les profils métaboliques observés après fermentation pouvaient être corrélés à la structure de la communauté bactérienne sélectionnée au cours de la fermentation, mais également, et de manière originale, à celle de l'inoculum. Afin de mettre en évidence de telles corrélations, des indicateurs biologiques basés sur l'affiliation bactérienne au niveau de la famille ont été proposés pour la première fois afin de simplifier les jeux de données issus du séquençage et ainsi mieux évaluer la structure de la communauté bactérienne. Une analyse canonique des corrélations réalisée entre ces indicateurs calculés à partir de l'abondance relative et de la diversité des séquences/OTUs identifiées dans l'inoculum, et les profils métaboliques a ainsi permis de mettre en évidence qu'un inoculum riche en *Clostridiaceae* permet de produire de l'hydrogène. Au contraire, lorsque des bactéries appartenant aux familles *Paenibacillaceae* and *Bacillaceae* sont présentes en majorité dans l'inoculum, un métabolisme orienté vers la production de lactate et non d'hydrogène est observé.

Ces indicateurs ont permis d'expliquer les métabolismes observés pour la fermentation du glucose. Comme uniquement deux structures de communauté bactérienne ont été utilisées pour l'analyse, l'ajout de résultats supplémentaires obtenus à partir d'autre inocula pourrait appuyer les corrélations observées ou mettre en évidence d'autres phénomènes permettant de lier la communauté bactérienne aux métabolismes fermentaires.

Ainsi, les deux premières parties de la thèse auront montré que la fermentation de substrats complexes entraînait la sélection de bactéries hydrolytiques, alors que, selon le type d'inoculum, la fermentation de sucre simple pouvait sélectionner des bactéries productrices d'hydrogène. Dans le cadre d'une production d'hydrogène à partir de résidus lignocellulosiques, il est nécessaire de sélectionner des communautés fermentaires à la fois composées de bactéries hydrolytiques et de bactéries productrices d'hydrogène. Cependant, les temps de fermentation requis pour hydrolyser les substrats complexes sont supérieurs à plusieurs semaines et sont par conséquent incompatibles avec les durées optimales pour la production d'hydrogène. En effet, l'émergence de bactéries consommatrice d'hydrogène apparaît sur le long terme.

La dernière partie de l'étude s'est donc penchée sur la sélection d'une communauté bactérienne présentant à la fois une activité hydrolytique et productrice d'hydrogène. Pour cela, la fermentation a été réalisée sur des polymères de glucose. La cellulose semi-amorphe (CSA) a été

choisie comme polymère pour la sélection de bactéries hydrolytiques, et le cellobiose pour la sélection de bactéries productrices d'hydrogène. Ce dernier a été préféré en tant que dimère au glucose pour conserver une activité d'hydrolyse. La fermentation de ces substrats a été étudiée sur 32 jours séparément, ainsi qu'en mélange pour observer l'effet de la co-fermentation sur la sélection bactérienne.

La fermentation sombre a été réalisée en quadruplicat en utilisant les boues activées fraîchement extraites de la station d'épuration comme inoculum, puisque ces communautés bactériennes ont montré un fort potentiel de production d'hydrogène. La production de gaz a été suivie régulièrement jusqu'à une production maximale d'H₂. A ce moment-là, 20 ml de milieu ont été prélevés pour l'analyse des métabolites et de la communauté bactérienne et 50 ml de nouveau milieu composé d'oligo-éléments ont été ajoutés dans tous les réacteurs, ainsi que 2 g_{DCO}·l⁻¹ de cellobiose dans les réacteurs contenant le dimère seul et en mélange. Cet ajout de cellobiose a été réalisé pour maintenir une activité de production d'hydrogène pendant la durée de l'expérience. Cinq cycles d'échantillonnage et d'alimentation ont été effectués, ce qui représente six cycles de production d'hydrogène.

Les résultats ont montré que sur cellobiose, des bactéries productrices d'hydrogène via la voie acétate/éthanol ont rapidement été sélectionnées, soit après 3 jours de culture uniquement. Sur CSA, ce sont des bactéries hydrolytiques appartenant aux familles *Ruminococcaceae* et *Lachnospiraceae* qui ont été sélectionnées dès les premiers jours de fermentation. Cependant, il a été observé qu'en fin d'expérience, les *Ruminococcaceae* étaient présents majoritairement dans tous les réacteurs, dont ceux fermentant le cellobiose. Ce résultat laisse supposer que sur le long terme, la fermentation d'oligo- ou polymères conduit à une sélection bactérienne vers une structure de communauté semblable, essentiellement composée de bactéries hydrolytiques, et ce afin d'hydrolyser la matière, indépendamment du degré de polymérisation. La sélection des *Ruminococcaceae* à partir de deux sources d'inoculum différentes, boues activées et digestat de fumier, suggère que la biomasse complexe oriente la sélection de la communauté bactérienne vers une structure capable d'une activité hydrolytique.

La co-fermentation du cellobiose avec la CSA a également conduit à la sélection de *Ruminococcaceae*. Cependant, la production d'hydrogène a pu être maintenue malgré la présence du polymère. De plus, en ne considérant que la part de DCO provenant du cellobiose, les volumes d'hydrogène étaient comparables, et même 1.4 fois supérieurs, à ceux observés lors de la fermentation du cellobiose seul. Dans ce cas, la production d'hydrogène était liée au maintien des

Enterobacteriaceae dont la présence était associée à la production d'hydrogène par la voie acétate/éthanol. La CSA a également été dégradée lors de la co-fermentation, mais en plus faible proportion que lorsqu'elle était fermentée seule. De ce fait, l'activité hydrolytique a bien été maintenue pendant la co-fermentation du cellobiose et de la cellulose, mais une inhibition due à la présence du cellobiose a été suggérée.

Cette thèse a permis d'apporter de nouvelles connaissances sur les interactions pouvant être observées pendant la fermentation sombre entre les communautés bactériennes, les métabolismes fermentaires et la composition du substrat. Il a ainsi été montré que la structure des communautés bactériennes était orientée par le type de substrat, soit sa composition moléculaire et son degré de polymérisation. La structure des communautés était plus sujette à des variations lorsque des sucres simples étaient utilisés. Dans le cas des sucres complexes, la communauté était toujours orientée vers la sélection de bactéries hydrolytiques, indépendamment de l'inoculum utilisé. Au regard de ces résultats, des perspectives de recherches ont pu être proposées.

Les changements de structure des communautés bactériennes pendant la fermentation sombre selon la composition des sucres solubles peut constituer un obstacle pour une production stable et continue d'hydrogène à l'échelle pilote et industrielle. En raison de la culture saisonnière, la composition des résidus agricoles en glucides varie pendant toute l'année, et peut donc entraîner des fluctuations de la production d'hydrogène au cours de la fermentation sombre. Cependant, la sélection spécifique de bactéries selon le type de sucre pourrait être utilisée pour gérer un consortium bactérien. Selon la communauté bactérienne fermentaire présente dans les réacteurs, l'introduction d'un sucre spécifique pourrait orienter la communauté vers une autre structure qui développera un métabolisme différent. Cela a été observé au cours de la co-fermentation du cellobiose et de la cellulose, où le métabolisme de production d'hydrogène a été maintenu par l'addition régulière de cellobiose. Cependant, le procédé fed-batch utilisé pour effectuer la sélection doit être amélioré pour éviter l'accumulation de métabolites dans la phase liquide, provoquant un changement du métabolisme et de la structure de la communauté bactérienne sur le long terme. La réalisation de la fermentation sombre en continu en réacteur à membrane ou en immobilisant les cultures mixtes peut être préférée, car, dans ces systèmes, les bactéries sont retenues dans le réacteur tandis que l'effluent contenant les co-produits de fermentation est évacué continuellement.

D'autres stratégies de gestion des communautés bactériennes par ajout de souches bactériennes pures, exogènes à la communauté initiale, ont également démontré des résultats positifs (Kótay et Das, 2010). Mais l'utilisation des cultures pures est coûteuse et le maintien des

bactéries exogènes dans un consortium naturel reste incertain. L'addition, dans les réacteurs, de sucres solubles provenant de déchets à la place de la culture pure de bactéries pourrait être une façon originale et moins coûteuse de contrôler le métabolisme fermentaire et d'améliorer ainsi la production d'hydrogène par fermentation sombre. Dans le cas de la production d'hydrogène à partir de polymères hétérogènes tels que la biomasse lignocellulosique, les fruits et les déchets végétaux peuvent représenter des sources de sucre simples afin d'éviter l'utilisation de sucres purs. Ces résidus constituent un gisement de déchets important puisque environ 20% des fruits et légumes récoltés sont gaspillés chaque année dans le monde (FAO, 2011).

Ces travaux ont montré la possibilité et l'intérêt de gérer les communautés bactériennes grâce à un contrôle des sucres disponibles. Toutefois, ces résultats doivent être consolidés par l'étude des interactions entre d'autres substrats et inocula. Pour cela, ces résultats suggèrent d'analyser la structure de la communauté au niveau de la famille, puisque ce rang taxonomique a été montré comme efficace pour expliquer la structure bactérienne tout en simplifiant les données.

Pour résumer, cette thèse a permis d'acquérir des informations importantes pour mieux comprendre les interactions entre le substrat, la communauté bactérienne et le métabolisme. Tous ces résultats peuvent être appliqués dans un avenir proche, peut aider à gérer les communautés bactériennes et ainsi mieux contrôler leurs métabolismes sur différents substrats.

Contents

INTRODUCTION	1
CHAPTER 1. LITERATURE REVIEW	7
1.1 HYDROGEN: A PROMISING AND CLEAN ENERGY VECTOR	9
1.2 PRODUCTION OF HYDROGEN	14
1.2.1 <i>General technologies producing hydrogen</i>	14
1.2.2 <i>Biological hydrogen production</i>	18
1.2.2.1 Water bio-photolysis	18
1.2.2.2 Photofermentation	21
1.2.2.3 Dark fermentation	22
1.2.2.4 Bio-electrochemical systems	24
1.3 DARK FERMENTATION	25
1.3.1 <i>Principle of dark fermentation</i>	25
1.3.2 <i>Metabolic pathways in dark fermentation</i>	28
1.3.3 <i>Coupling dark fermentation with other biological processes to improve hydrogen yield...</i>	32
1.3.4 <i>Microbiology of dark fermentation</i>	34
1.3.4.1 Hydrogen-producing bacteria	34
1.3.4.2 Characteristics of microbial ecology in mixed cultures used in dark fermentation	36
1.4 LIGNOCELLULOSIC RESIDUES AS A RESOURCE FOR BIOLOGICAL HYDROGEN PRODUCTION.	38
1.4.1 <i>Composition of lignocellulose</i>	38
1.4.2 <i>Fermentative biological degradation</i>	41
1.4.3 <i>Impact of lignocellulosic substrate on dark fermentation</i>	44
1.4.3.1 The substrate composition conduce to different hydrogen production potential	44
1.4.3.2 Use of pretreatment to increase hydrogen production yield	49
1.5 IMPORTANCE OF MICROBIAL COMMUNITY ON THE DARK FERMENTATION PROCESSES IN MIXED CULTURE	54
1.5.1 <i>Evidence of the influence of inoculum origin on dark fermentation</i>	54
1.5.2 <i>Analytical technics to understand structure-function relationship in mixed culture</i>	58
1.6 CONCLUSION AND AIM OF THE THESIS	59
CHAPTER 2. MATERIALS AND METHODS	61
2.1 BIOLOGICAL PROCESS FOR HYDROGEN PRODUCTION	63
2.1.1 <i>Source of mixed microbial communities</i>	63
2.1.2 <i>Biohydrogen production in batch tests</i>	64
2.1.3 <i>Dark fermentation in fed-batch reactor</i>	65
2.1.4 <i>Summary of the experimental conditions with references to chapter numbering</i>	66
2.2 BIOCHEMICAL AND BIOLOGICAL ANALYSIS	67

2.2.1	<i>Biochemical analyses</i>	67
2.2.1.1	Total Solids and Volatile Solids, and Chemical Oxygen Demand analysis.....	67
2.2.1.2	Analyses of gas and metabolites produced.....	67
2.2.2	<i>Biological analyses</i>	68
2.2.2.1	Sample collection.....	68
2.2.2.2	DNA extraction and purification.....	68
2.2.2.3	Polymerase chain reaction (PCR) amplification and Illumina Sequencing (High-throughput amplicon sequencing).....	69
2.3	DATA ANALYSES.....	70
2.3.1	<i>Determination of kinetic parameters of the H₂ production</i>	70
2.3.2	<i>Multivariate analysis performed on metabolic patterns and bacterial community structure – Principal Component Analysis (PCA)</i>	70
2.3.3	<i>Identification of bacterial community structure gradient with Nonmetric multidimensional scaling (NMDS)</i>	71
2.3.4	<i>Searching for correlations between metabolic patterns and bacterial families present in inocula and selected mixed cultures</i>	72
2.3.5	<i>Summary of statistical tests used in this thesis</i>	72
	CHAPTER 3. IMPACT OF THE SUBSTRATE TYPE ON FERMENTATION	75
4.1	CONTEXT AND OBJECTIVES.....	77
4.2	IMPACT OF SUBSTRATE COMPOSITION ON DARK FERMENTATION PERFORMED INOCULATED WITH A FRESH DIGESTED MANURE (FM).....	79
4.2.1	<i>Hydrogen production performances</i>	79
4.2.2	<i>Other fermentation metabolites</i>	82
4.2.3	<i>Strong selection in microbial communities</i>	85
4.2.4	<i>Conclusion</i>	92
4.3	IMPACT OF SUBSTRATE COMPOSITION ON DARK FERMENTATION PERFORMED BY STORED DIGESTED MANURE INOCULUM (StM).....	93
4.3.1	<i>Impact of inoculum storage on hydrogen and other metabolites production</i>	93
4.3.2	<i>Bacterial communities structure developed in stored inoculum</i>	96
4.3.3	<i>Comparison of bacterial community culture selected on fresh and stored inocula for the different substrates</i>	101
4.4	CONCLUSION.....	103
	CHAPTER 4. RELATIONSHIPS BETWEEN INITIAL AND FINAL BACTERIAL COMMUNITY STRUCTURES, AND METABOLISMS IDENTIFIED AFTER DARK FERMENTATION	105
4.1	CONTEXT AND OBJECTIVES.....	107
4.2	IMPACT OF THE TYPE OF INOCULUM USED ON THE HYDROGEN PRODUCTION.....	108

4.3	DIFFERENT METABOLIC PATTERNS DEVELOPED BY THE FIVE INOCULA DURING THE BIOLOGICAL PROCESS.	111
4.4	STRUCTURES OF BACTERIAL COMMUNITY INTRODUCED IN REACTOR AND SELECTED FERMENTATIVE COMMUNITY. 114	
4.5	CORRELATION BETWEEN INITIAL BACTERIAL STRUCTURE AND METABOLIC PERFORMANCES	118
4.6	CONCLUSION	124
CHAPTER 5. SELECTION OF HYDROLYTIC CULTURES ABLE TO PERFORM HYDROGEN PRODUCTION		
BY DARK FERMENTATION..... 125		
5.1	CONTEXT AND OBJECTIVE	127
5.2	ANALYSIS OF BIOLOGICAL ACTIVITY DURING THE EXPERIMENT	128
5.2.1	<i>Increase of substrate conversion yield during fed-batch dark fermentation</i>	<i>128</i>
5.2.2	<i>Accumulation of hydrogen along the fed-batch cycles and associated metabolic patterns</i> <i>130</i>	
5.3	BACTERIAL COMMUNITIES SELECTED IN EACH REACTOR ALONG THE EXPERIMENT.	135
5.3.1	<i>Structure of the initial bacterial community used as inoculum</i>	<i>135</i>
5.3.2	<i>Bacterial community change along fed-batch cycles.....</i>	<i>137</i>
5.3.3	<i>Link between metabolic patterns and selected community structures after fed-batch cycles</i> <i>140</i>	
5.4	CONCLUSION	144
GENERAL CONCLUSION AND OUTLOOKS 147		
REFERENCES 155		
ANNEXES 185		

List of figures

Figure 1: Scheme of the experimental strategy developed during this thesis to explore the tri-interaction present in dark fermentation process between substrate composition, bacterial community structure and fermentative metabolic patterns.	5
Figure 1.1-1: Main hydrogen consuming industries (adapted from Chaubey et al., 2013)	10
Figure 1.1-2: Toyota clean hydrogen production and supply chain (Kanagawa Prefectural Government et al., 2016)	12
Figure 1.1-3: Countries taking part of H2ME project and infrastructures. Colours and symbols correspond to countries concerned by the project (■), observed countries (■), Refuelling stations (●) and main European roads (—). Source: H2ME roadmap (H2ME, 2015).	13
Figure 1.2-1: scenario of hydrogen producing method imagined by the U.S. Department of Energy (DOE) (FreedomCAR & Fuel Partnership, 2009)	18
Figure 1.2-2: Example of direct bio-photolysis for the production of hydrogen in microalgae .	19
Figure 1.2-3: Hydrogen production in purple non-sulphur bacteria, adapted from Deo <i>et al.</i> (2012)	21
Figure 1.2-4: General scheme of dark fermentation, adapted from (Adapted from Latrille et al., 2011).....	23
Figure 1.2-5: General scheme of microbial electrolysis cell (MEC). Figure from the U.S. DRIVE Patnership (2013)	24
Figure 1.3-1: Enzymatic system to produce hydrogen developed by a) enteric bacteria from formate conversion, and by strict anaerobic bacteria from b) NADH-dependent hydrolase, c) bifurcating NADH-Fd dependent hydrolase, d) Fd-dependent hydrogenase with Fd reduction from NADH oxidation, and e) Fd-dependent hydrolase with production of NADH.	28
Figure 1.3-2: General and simplified fermentative metabolic pathway observed in anaerobic conditions from glucose. Green and orange arrows correspond to an accumulation and consumption of reduced intermediates, respectively, and the production of hydrogen through formate pathway.	

The red block corresponds to the principal pathway of direct hydrogen consumption. The reaction noted ① is the initiated butyrate production pathway by acetate consumption..... 31

Figure 1.3-3: Coupling all biological hydrogen production systems to increase hydrogen yields. 32

Figure 1.4-1: Structural composition of lignocellulosic biomass (from Barakat et al., 2013).... 39

Figure 1.4-2: Primary lignin monomers and their corresponding form in lignin (from Wong, 2009)..... 40

Figure 1.4-3: Main residues composing the hemicellulosic fraction (extracted from Kögel-Knabner, 2002) 40

Figure 1.4-4: Chemical structure of cellulose (from Estela and Luis, 2013)..... 41

Figure 1.4-5: Metabolic pathway to convert hemicelluloses and cellulose through dark fermentation. 43

Figure 1.4-6: Production of hydrogen depending on the lignocellulosic substrate obtained in the literature. Blue bars represent pure commercial carbohydrates and green bars are natural lignocellulosic biomass. 48

Figure 4.2-1: Production of hydrogen analysed for each substrate during the experiment mlH₂.g⁻¹ of initial substrate eq.COD for a) glucose (●), cellobiose (■), arabinose (▲), xylose (▼), xylan (◆), and wheat straw (★), and b) for MCC (●). 80

Figure 4.2-2: Principal component analysis done on metabolite production. Symbols correspond to each substrate, Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★). 84

Figure 4.2-3: Principal component analysis (PCA) done on OTUs after Hellinger transformation. Symbols correspond to each substrate, Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★). 86

Figure 4.2-4: Major OTUs identified in reactors for each substrate (relative abundance >5%). White colour corresponds to absence of identified OTU, light blue a low relative abundance, and black relative abundance close to the maximum measured (38%) 87

Figure 4.2-5: Principal component analysis (PCA) ordination plot of major bacteria composing the sample bacterial community (a) with metabolite variables as predictors onto the ordination (b) analysed by the *envfit* function (permutations 9999, R package *vegan*). Symbols correspond to substrates: Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★); and to number of Operation Taxonomic Unit (OTU, †). Stars following metabolite name represent the significance of the fit (**<0.001; *<0.01, <0.05). 88

Figure 4.3-1: Metabolites patterns developed for each substrate by a) the inoculum without storage and b) the stored inoculum in $g_{ed. COD} \cdot 100g_{initial substrate eq. COD}^{-1}$. Error bars represent the standard deviations calculated between the four replicates. Values above each bar correspond to the percentage of COD converted into metabolites at the end of the experiments \pm standard deviation observed between each replicate. 95

Figure 4.3-2: PCA realised on OTUs after Hellinger transformation. Symbols correspond to each substrate, Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★). 97

Figure 4.3-3: Heatmap representing the average of relative abundance of the major OTUs identified at the end of the fermentation of each sugar. White colour corresponds to non-identification of the OTU, light blue and black to low and high relative abundance, respectively (max=35%). OTUs that were identified in bacterial community of both FM and StM inocula are framed in red. 99

Figure 4.3-4: PCA performed with sequencing data of both fresh (white symbols) and stored inocula (black symbols). The forms of symbols correspond to the different substrates used for the fermentation, i.e. glucose (○), cellobiose (□), MCC (○), arabinose (△), xylose (▽), xylan (◇), and wheat straw (★). 102

Figure 5.2-1: Amount of hydrogen accumulated during glucose fermentation. Symbols correspond to the type of inoculum used, i.e. FAS (●), SAS (■), FSAS (◆), FM (□), StM (▲). 109

Figure 5.3-1: Metabolites produced during dark fermentation using the five inocula with a) the quantity of each product in $mg_{metabolite eq. COD} \cdot g_{initial COD}^{-1}$ and b) a graphical representation of their relative proportion for each inoculum. In equivalent COD 112

Figure 5.3-2: Principal Components 1, 2 (a) and 1, 3 (b) for the final metabolite composition (in $\text{gCOD produced} \cdot \text{g}^{-1} \text{COD converted}$). In all panels, data corresponding to the five inocula are differentiated by their symbol shape: FM (▲), StM (▼), FAS (●), SAS (■) and FSAS (◆). 113

Figure 5.4-1: Principal component analysis (PCA) performed on community structure as absolute abundance of unique sequence types. In all panels, data corresponding to the five inocula are differentiated by their symbol shape: FM (▲), StM (▼), FAS (●), SAS (■) and FSAS (◆). Filled shapes (e.g. ●) indicate final compositions after fermentation. Additionally on panels c and d, the inocula structure before fermentation is indicated as light symbols (e.g. ○). 115

Figure 5.4-2: Heatmap representing the relative abundance of the major sequences (>5%) identified in reactors at the end of the fermentation of glucose with 5 inocula. 116

Figure 5.5-1: Major correlations found by rCCA by confronting indexes calculated from a) sequences identified from inoculum and b) sequences identified in selected communities, to metabolites produced during dark fermentation. This figure is only a sample of the total indexes tested. The full heatmaps are presented in Annex I and Annex II. 122

Figure 6.1-1: Fed-batch experimental procedure performed in the present chapter. 128

Figure 6.2-1: Cumulative amount of hydrogen obtained along the different fed-batch cycles for SAC (●); Cellobiose (●); Mix (●) and Mix if considering only the COD from cellobiose (○), in $\text{ml}_{\text{H}_2} \cdot \text{g}^{-1} \text{initial COD}$. Error bars correspond to the standard deviation observed between four replicate. Dotted lines represent a new cycle start, i.e. the insertion cellobiose in the medium. 131

Figure 6.2-2: Relative proportion of metabolites identified at the end of each fed-batch cycle (C1 to C6) in equivalent COD for reactors fed with cellobiose (a), SAC (b) and mix of these two substrates (c). Error bars correspond to the difference measured between replicates. 133

Figure 6.3-1: PCA performed on initial bacterial community structure inoculated in reactors. Green and orange symbols represent samples used in Chapter 5, i.e. FAS (●), SAS (■), FSAS (◆), FM (▲), StM (▼). Blue stars correspond to the inoculum community structure used in the present chapter (★). 136

Figure 6.3-2: Graphical representation of relative abundance of main fermentative bacteria families identified in dark fermentation at the end of each fed-batch cycle (C1 to C6) for the three types of reactors. White colour corresponds to relative abundance below to 5%. 138

Figure 6.3-3: Ordination graph for a two-dimensional NMDS of the identified sequences based on the Bray-Curtis distance. The first plot (a) represents similarities between samples along the different cycles (number below symbols) for reactors fed with cellobiose (●), with SAC (▲), and with a mix of these two substrates (◆). The second graph (b) represents similarities between identified sequences affiliated to *Firmicutes* (●), *Proteobacteria* (●) and other taxonomic phyla (●)..... 139

Figure 6.3-4: Regularised Canonical Correlation Analysis (rCCA) performed on calculated indicators..... 142

List of tables

Table 1.1-1: Energetic content of usual energetic vectors in MJ/kg (adapted from Dutta, 2014)	10
Table 1.2-1: techno-economic analysis of existing technologies to produce hydrogen. Information are extracted from (1) the US DRIVE Hydrogen Production Technical Team (HPTT) roadmap (2013), (2) Megret <i>et al.</i> (2015) and Parthasarathy <i>et al.</i> (2014)	15
Table 1.4-1: Literature review done on the production of hydrogen according different lignocellulosic substrates in batch reactor. The first block is pure commercial carbohydrate ranked by their degree of polymerisation. The second block is lignocellulosic biomass from waste or energy crops in alphabetic order. Hydrogen production is expressed in the following unit: a) ml H ₂ .g _{TS} ⁻¹ ; b) ml H ₂ .g _{VS} ⁻¹ ; c) ml H ₂ .g _{TVS} ⁻¹ ; d) ml H ₂ .g ⁻¹ _{initial substrate}	44
Table 1.4-2: Enhancement of biohydrogen production from different agricultural residues and operative conditions. Hydrogen production is expressed in the following unit: a) ml H ₂ .g _{COD} ⁻¹ ; b) ml H ₂ .g _{VS} ⁻¹ ; c) ml H ₂ .g _{TVS} ⁻¹ ; d) ml H ₂ .g ⁻¹ _{substrate} . (* enzymes applied after alkali treatment: Cellulase, β-glucosidase and xylanase : 50, 25, and 50 units.g ⁻¹ of TS)	50
Table 1.5-1: Results of hydrogen yield, metabolic patterns and identified bacteria obtained from dark fermentation performed on different inocula and substrates in the literature. Metabolites correspond to acetate (HAc); butyrate (HBut); caproate (HCap); ethanol (EtOH); lactate (HLac); propionate (Hpr); formate (Hfor); valerate (HVal) and succinate (HSuc). Hydrogen production are expressed as a) mol _{H2} .mol ⁻¹ _{substrate} ; b) mmol _{H2} .g ⁻¹ _{VS} ; c) mmol _{H2} .g ⁻¹ _{substrate} ; d) mmol _{H2} .g ⁻¹ _{TVS} ; e) mmol _{H2} .g ⁻¹ _{DCO}	56
Table 2.1-1: Summary of the different conditions used for the experiments performed in the thesis chapters.	66
Table 2.3-1: Summary of statistical test realised in for the thesis.	73
Table 3.2-1: Kinetic parameters of hydrogen production in batch tests according to the initial substrate concentration determined from modified Gompertz equation with P the maximum cumulative hydrogen production (mlH ₂ .g-1eq initial COD), R _m the maximum hydrogen production rate (mlH ₂ .g-1eq initial COD.d-1), λ the lag phase (days) and R ² the coefficient of determination of	

the model. Values correspond to the average of four replicates ± standard deviation observed between these replicates. Values in % in the H² yield column correspond to the proportion of the maximum theoretical value suggested by Hawkes et al. (2007), i.e 2.5 molH₂.mol⁻¹ degraded substrate in glucose equivalent..... 81

Table 3.2-2: Metabolites accumulated at the end of the fermentation tests (in mg_{product eq. COD}·g_{initial substrate eq. COD}⁻¹.) Values correspond to the average of four replicates ± standard deviation. Values in italic and in parenthesis correspond to the proportion (in %) of each product among all the metabolites produced in a same batch test ± standard deviation. 83

Table 3.2-3: Hydrogen yields, metabolic patterns and main identified bacteria obtained from dark fermentation performed on different inocula and substrates in the literature. Units of hydrogen yield, H₂ column, correspond to a) mol_{H₂}·mol⁻¹_{substrate}, b) mmol_{H₂}·g⁻¹_{VS}, c) mmol_{H₂}·g⁻¹_{substrate}, d) mmol_{H₂}·g⁻¹_{TVS} and e) mmol_{H₂}./g⁻¹_{DCO}. Metabolites are expressed in % of COD converted and abbreviations correspond to Acetate (HAc), Butyrate (HBut); Caproate (HCap), Ethanol (EtOH), Lactate (HLac), Propionate (Hpr), Formate (Hfor), Valerate (HVal) and Succinate (HSuc). 90

Table 3.3-1: Modified Gompertz parameters calculated for each substrate and for the two inocula, before (italic, FM) and after storage (bold underline, StM), with P the maximum cumulative hydrogen production (mlH₂.g-1eq initial COD), R_m the maximum hydrogen production rate (mlH₂.g-1eq initial COD.d-1) and λ the lag phase (days). Values correspond to the average of four replicates ± standard deviation observed between these replicates..... 93

Table 4.2-1: Kinetic parameters of hydrogen production in batch tests according to the initial substrate concentration determined from modified Gompertz equation with P the maximum cumulative hydrogen production (mlH₂.g-1eq initial COD), R_m the maximum hydrogen production rate (mlH₂.g-1eq initial COD.d-1), λ the lag phase (days) and R² the coefficient of determination of the model. Values correspond to the average of four replicates ± standard deviation observed between these replicates. Values in % in the H² yield column correspond to the proportion of the maximum theoretical value suggested by Hawkes et al. (2007), i.e 2.5 molH₂.mol⁻¹ degraded substrate in glucose equivalent..... 110

Table 4.5-1: Result of Mantel test after 9999 permutations. RM correspond to Mantel statistic value, α is the risk at 1%..... 118

Table 4.5-2: Description of the biological indexes calculated from the sequences identified by high throughput sequence (Illumina MiSeq). These indexes were both calculated for bacterial initial and final communities..... 120

Table 5.2-1: Quantity of COD recovered under the form of metabolites at the end of each cycle (C1 to C6) per gram of initial COD introduced for the three types of reactor and MIX reactor when only considering COD from cellobiose (fourth line). Standard deviation represents the difference between four replicates 129

Table 5.3-1: Description of the main biological indexes calculated from the sequences identified by high throughput sequence (Illumina MiSeq). 141

Table of abbreviations

Abbreviation	English translation	Traduction française
CB	Cellobiose	Cellobiose
COD	Chemical Oxygen Demand	Demande chimique en oxygène
EtOH	Ethanol	Éthanol
FAS	Fresh Aerobic Sludge	Boues activées fraîches
FAS-<i>Ap</i>	Fresh Aerobic Sludge - sampled in April	Boues activées fraîches - échantillonnées en avril
FM	Fresh Manure digestate	Digestat de fumier frais
FSAS	Fresh and Settle Aerobic Sludge	Boues activées fraîches après décantation
GHG	Greenhouse gas	Gas à effet de serre
H₂	Hydrogen	Hydrogène
H2ME	Hydrogen Mobility Europe	Hydrogen Mobilité Europe
HAc	Acetate	Acétate
HBut	Butyrate	Butyrate
HCap	Caproate	Caproate
HFor	Formate	Formate
HiBut	Isobutyrate	Isobutyrate
HiVal	Isovalerate	Isovalérate
HLac	Lactate	Lactate
HPr	Propionate	Propionate
HRS	Hydrogen Refueling Station	Station service d'hydrogène
HSucc	Succinate	Succinate
HVal	Valerate	Valérate
LGP	Liquified gas petroleum	Gas de pétrole liquéfié
MCC	Micro-crystalline cellulose	Cellulose micro-crystalline
PNSB	Purple non-sulphur bacteria	Bactérie pourpre non-sulfureuse
SAC	Semi-amorphous cellulose	Cellulose semi-amorphe
SAS	Stored Aerobic Sludge	Boues activées stockées
StM	Stored Manure digestate	Digestat de fumier stocké
TS	Total solid	Matière sèche
VS	Volatile solid	Matière volatile

Articles and communications

Publications scientifiques et communications orales

Peer-reviewed articles

Chatellard, L., Marone, A., Carrère, H., Trably, E., 2017. Trends and Challenges in Biohydrogen Production from Agricultural Waste, in: Biohydrogen Production: Sustainability of Current Technology and Future Perspective. Springer India, New Delhi, pp. 69–95. doi:10.1007/978-81-322-3577-4_4

Chatellard, L., Trably, E., Carrère, H., 2016. The type of carbohydrates specifically selects microbial community structures and fermentation patterns. *Bioresour. Technol.* 221, 541–549. doi:10.1016/j.biortech.2016.09.084

International communications

Chatellard, L., Trably, E., Milferstedt, K., Steyer, J.P., Carrère, H., Production of chemicals and energy carrier from lignocellulosic compounds by dark fermentation: impact of inoculum history and nature of sugars. *Biorefinery for Food, Fuels and Materials*, Montpellier - France, 15-17th June, 2015

Chatellard, L., Trably, E., Carrère, H., Cross-impact of initial sugars type and microbial community origin and history on fermentative production of biohydrogen and biomolecules from lignocellulosic biomass. *21th World Hydrogen Energy Conference 2016*, Zaragoza, Spain, 13-16th June, 2016

National communications

Chatellard, L., Trably, E., Milferstedt, K., Steyer, J.P., Carrère, H., Production de biohydrogène et de molécules plateformes à partir de résidus lignocellulosiques par fermentation sombre : Impact de l'histoire de l'inoculum et de la nature des sucres sur le procédé biologique. *Séminaire d'ouverture du GDR Symbiose*, Nantes, 8-10 April 2015

Introduction

Lifestyle in modern societies is based on industrial systems with activities generating large amounts of greenhouse gases that have been pointed as the main cause of global warming (Cook et al., 2016). Overproduction of goods to satisfy an ever-growing demand has led to natural resource depletion and accumulation of waste that contribute to degrade the environment (Gao and Tian, 2016; Toth and Szigeti, 2016). Awareness that human activities significantly contribute to global warming started in the seventies, leading governments to engage in environmental care, up to the recent signature of Paris agreement (UNFCCC. Conference of the Parties (COP), 2015). Waste management and energetic transition to renewable and clean energy are ones of the 21st century priorities to preserve Nature while maintaining Human activities.

On contrary to fossil fuels, renewable energy can be obtained from natural resources that are constantly replenished on human timescale. They can be produced from various sources such as hydro, solar, wind, tidal, geothermal and biomass, and emit less pollution than fossil fuels. Research and development of renewable energies are nowadays supported by various funding. As an example, Europe Union's Research and Innovation Programme Horizon 2020 will provide up to €5.931 billion in funding renewable energy projects between 2014 and 2020 (European Commission, 2016).

Among the energies substituting to fossil fuels, biomass resources represent about two thirds of the renewable energies consumed in EU in 2012. Biomass can be defined as the organic matter composing plants and animal residues. Historically, wood has been used for millennia to provide light and heat. The production of bio-ethanol by fermentation of carbohydrates from food crops was then developed, first for urban lighting and household heating and then for internal combustible engine, representing the first generation of biofuels (Kolb, 2014). Dedicate food crops to energy production is controversial in the current context where one human out of nine suffers of famine, and also because of their contribution to deforestation and loss of diversity (Carrquiry et al., 2011).

These concerns have led to the development of second generation biofuels based on non-food biomass such as agricultural waste. This latter corresponds to the residual organic part of agricultural biomass that cannot be used for human feed. Conversion of these residues into energy before recycling could be a solution to answer to both organic waste management and renewable energy production. In the sole case of France, 16% of straw, equivalent to 4 million tons of biomass, resulting from cereals production could be converted into energy without agronomic impact (Gagnaire et al., 2006). Agricultural waste is mainly composed of lignocellulose, a complex and heterogeneous matrix containing 66% of carbohydrates under polymeric forms. In biological processes, biomass hydrolysis generates simple carbohydrates that can be further converted into bio-energy, in a similar way as when carbohydrates are extracted from food crops.

As all organic matter, the main four elements composing lignocellulose are carbon, oxygen, nitrogen and hydrogen. This last compound represents the smallest chemical element widely distributed on earth, that once combined to another hydrogen atom form gaseous dihydrogen, commonly named hydrogen (H_2). H_2 is an interesting energetic vector since its energy content per mass is higher than most of the other usual energy vectors (120 kJ.g^{-1}) and its combustion only produces water, making it as a serious candidate to replace pollutant fossil fuels like petrol.

Hydrogen can be produced by a biological process called dark fermentation which is based on the activity of anaerobic microorganisms that are able to degrade and convert organic matter into hydrogen. These microorganisms are found in natural and anthropogenic environments such as marshes, sediments, manure or sewage sludge, and are thus easily available.

Hydrogen production by conversion of simple sugars by dark fermentation has already demonstrated the potential of this technic, with a maximum of 2.5 moles of H_2 obtained per mole of glucose. In the case of more complex biomass such as lignocellulose, lower amounts of hydrogen are generally obtained since a first hydrolytic step is required to release monomers of carbohydrates that can then be converted into hydrogen. The heterogeneous composition of lignocellulose requires the use of different types of hydrolytic enzymes to fully degrade all the polymers. Such complex enzymatic potential can be produced by consortia of microorganisms which increase biomass degradation thanks to complementary enzymatic functions (Jobard et al., 2014). In this context, the use of complex microbial consortia presents the advantages to not require sterile conditions and easily adapt to culture condition changes. However, the hydrogen produced during the conversion of organic matter only represents 20% of the COD composing the substrate. The remaining 80% are found in the forms of fermentative metabolites co-produced during the dark fermentation process. In addition, hydrogen non-producing microorganisms present in mixed cultures can convert the substrate into other metabolites than hydrogen, depending on their own metabolism. The presence of hydrogen-consuming microorganisms might also considerably reduce the amounts of cumulated hydrogen. Up to now, dynamics of microbial communities in dark fermentation systems regarding the substrate composition and related fermentative metabolisms is still misunderstood.

The recent development and emergence of new biomolecular technologies to study microbial community structures in complex environments offer the possibility to better understand the fermentation process to further propose solutions to improve the production of hydrogen. This thesis is part of this approach, focusing on the tri-interaction that is present between bacterial consortia, substrate composition and fermentative metabolism. For this, three specific questions will be developed, as illustrated in Figure 1:

1. How does the type of sugars drive the bacterial selection and the metabolic patterns?
2. How does the initial structure of the bacterial community impact the dark fermentation mechanism?
3. How to manage bacterial community structure to improve the conversion of the substrate and the production of hydrogen?

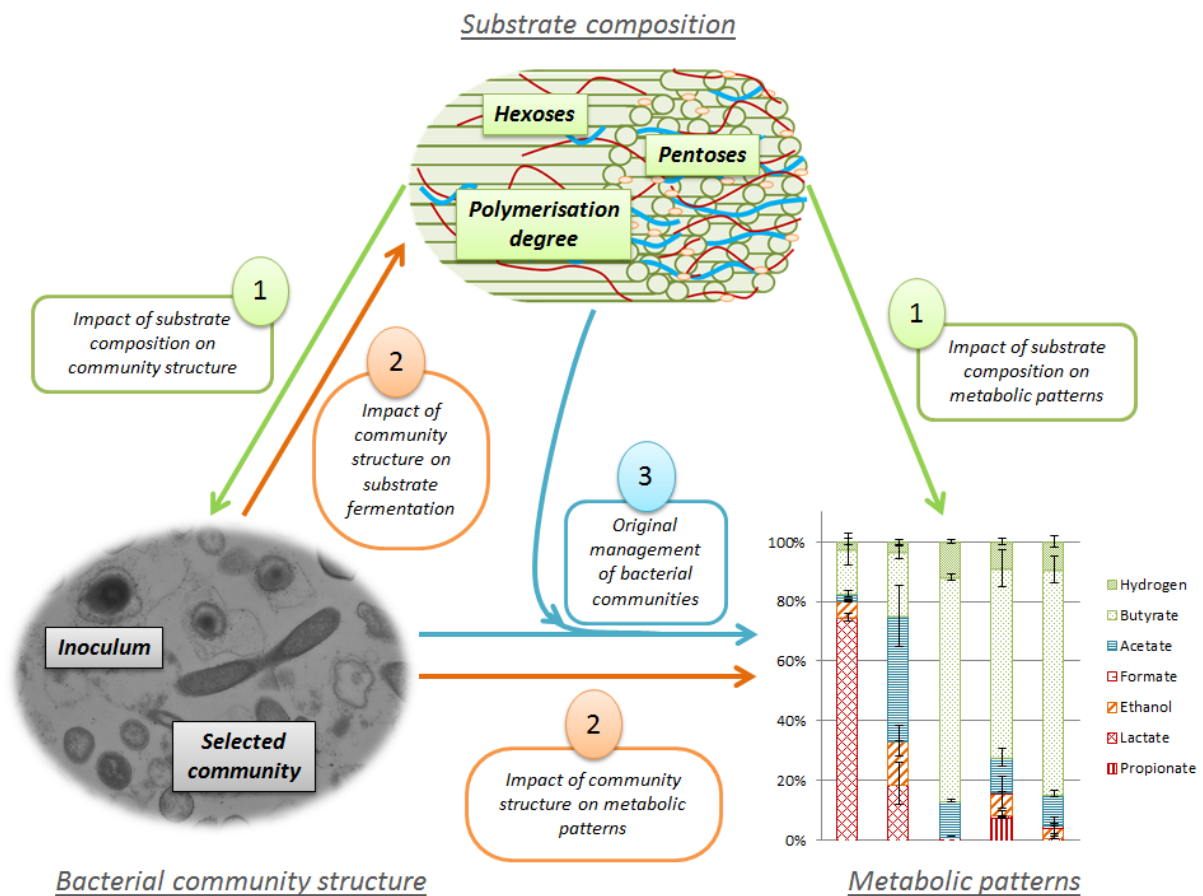


Figure 1: Scheme of the experimental strategy developed during this thesis to explore the tri-interaction present in dark fermentation process between substrate composition, bacterial community structure and fermentative metabolic patterns.

This work is presented in five chapters as follows.

The first section is devoted to a literature review on hydrogen production by dark fermentation of lignocellulosic biomass. It consists in a brief presentation of hydrogen uses and the general ways of production, a description of dark fermentation and lignocellulosic biomass degradation, and finally the importance of microbial community structures on the dark fermentation process in mixed cultures is presented.

The second chapter presents the experimental and analytical methods used in this work.

The third chapter presents the results and discussions focussed on the impact of substrate composition on dark fermentation. In this study, dark fermentation was performed on seven types of substrates representing the main carbohydrate fractions of lignocellulosic materials, i.e. glucose, cellobiose and micro-crystalline cellulose to represent the cellulosic fraction, arabinose, xylose and xylan for the hemicellulosic fraction, and wheat straw as model of lignocellulosic biomass. The impact of both sugar composition and degree of polymerisation on bacterial community structure and metabolic patterns was analysed.

Then, the influence of different bacterial community structures on dark fermentation mechanisms, i.e. substrate conversion and metabolisms, was studied on glucose in the fourth chapter. Manure digestate and sewage sludge were chose as microbial communities. A statistical approach was proposed to link final and initial bacterial community structure to fermentative metabolisms.

The fifth chapter was focused on the selection of bacterial community structure that efficiently hydrolyses polymeric carbohydrates while producing comparable quantities of hydrogen to simple sugars fermentation. The selection carried out in fed-batch reactors was performed on a mixture of two glucose polymers, i.e. semi-amorphous cellulose for the selection of hydrolytic bacteria, and cellobiose for the selection of hydrogen producing bacteria.

Finally the last section corresponds to an overall discussion of the results, and several perspectives for further research work are proposed.

Chapter 1. Literature review

1.1 Hydrogen: a promising and clean energy vector

Hydrogen has become an interesting energy vector for the last 30 years because of concerns rising about fossil fuel depletion and global warming. In the near future, fossil fuel could be substituted by hydrogen thanks to its high energy value. Nonetheless, the use of hydrogen has already a long history with first uses in 1807 in the first internal combustibile engine build by François Issac de Rivaz, Swiss inventor. He was followed by Etienne Lenoir (France) in 1859 who invented a car using hydrogen generated by water electrolysis as combustibile (Eckermann, 2001). Since these years, many other examples of hydrogen uses in vehicles have been reported but the Hindenburg disaster developed to people a non-justified fear for this gas. At that time, and because of its low density, hydrogen was the ideal gas to be utilised in airships. But during the first course of the largest ever-built airship Hindenburg, a storm ignited the material containing hydrogen, causing a gas explosion and the death of 33 people on board (Liao and Pasternak, 2009). Although the investigation proved the type of H₂ containing-material was the cause of the accident, hydrogen has remained to people the main cause of the tragedy.

Today, hydrogen is mainly used in chemical and petrochemical industries for non-energetic uses (Figure 1.1-1). The production of ammonia represents a large part of hydrogen consumption since more than 80 Mt of ammonia (i.e. 80% of uses) were used as fertilizer in agriculture in 2013, representing 30-50% of the total hydrogen production (Megret et al., 2015). Production of ammonia consists in hydrogenation of nitrogen at high temperature and high pressure, through the Haber-Bosch process. Current ammonia production plants produce more than 1,500t a day, corresponding to 265t of hydrogen consumption (Andersson and Lundgren, 2014). Oil refining is the second most important hydrogen consuming sector. During this process, dihydrogen is used to remove sulphur, nitrogen and metal compounds, and to branch alkanes which increase the economic value of petroleum distillates (Ramachandran and Menon, 1998). H₂ is also used to purify gases by reacting with residual oxygen to form water that is easier to be removed. The production of chemicals such as methanol also requires hydrogen since the solvent is produced by reaction of carbon monoxide with hydrogen. The resulting methanol is then used as feedstock in plastic manufacture and can also serve as fuel thanks to its combustion properties (Van-Dal and Bouallou, 2013). In minor proportion, hydrogen is used in metallurgical, electronical and glass industries to remove oxygen and prevent compounds oxidation (Ramachandran and Menon, 1998). In agroindustry, hydrogenation is also used to reduce the degree of unsaturation of vegetable oils. The resulting oil is less sensitive to oxidation and can be more easily converted to solid products such as margarine (Larqué et al., 2001). However,

this process also converts the initial cis-forms fatty acid into trans-forms molecules which are known to provoke cardiovascular diseases.

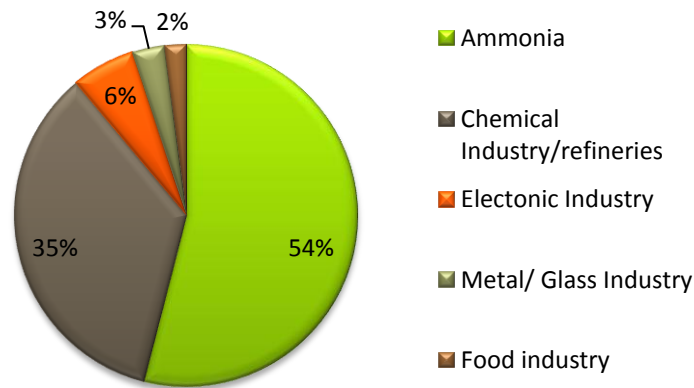


Figure 1.1-1: Main hydrogen consuming industries (adapted from Chaubey et al., 2013)

For the past decades, environmental care has conducted to reconsider hydrogen as a potential energetic vector. In addition to only produce water when burning, the H₂ gas has the highest energetic value per mass content when compared to other energy sources as presented in Table 1.1-1 (Latrille et al., 2011). Its energetic value is more than twice higher than natural gas or propane and gasoline, but also seven times higher than wood (Dutta, 2014). Uranium remains the sole element with a higher energetic density but its use causes diverse environmental problems such as production of untreated nuclear waste. Moreover, previous nuclear disasters such as Chernobyl (Ukraine in 1986) and more recently Fukushima (Japan in 2011) have led several governments to reconsider or even abandon this energy source (Hayashi and Hughes, 2013). Hence, Japan has decided to turn its nuclear energy into clean hydrogen and estimates that they can turn to a full hydrogen society before 2040, with a target in 2020 to demonstrate its feasibility during Tokyo Olympic and Paralympic games (Tokyo metropolitan Government, 2016).

Table 1.1-1: Energetic content of usual energetic vectors in MJ/kg (adapted from Dutta, 2014)

Hydrogen	Uranium	Liquefied natural gas	Propane	Methane	Gasoline	Ethanol	Methanol	Wood (dry)
120	417,600	54.4	49.6	46.8	46.8	29.6	19.7	16.2

In a future hydrogen society, hydrogen shall be produced from different sources to supply diverse industry, residential, commercial and transportation applications, and will be provided through hydrogen networks (Marbán and Valdés-Solís, 2007). Improvement of hydrogen storage

technologies permit to use the gas to store overproduction of electricity obtained through renewable process. Combination of “power-to-gas” and “gas-to-power” systems represent a solution to answer to energy production fluctuation observed when using renewable energy source.

Since hydrogen is now considered as a potential energy for the near future, expansion of hydrogen society model will greatly depend on the other fuel energy markets (Ball and Weeda, 2015). Up to now, hydrogen energy is not competitive to fossil and bio-fuels, and according to some reports, H₂ will only be competitive when fossil fuels will be depleted or when hydrogen price will decrease below oil (Midilli et al., 2005). Recently, the US department of energy (DOE) published a report with estimation of hydrogen cost depending on the technology used within the next four years. They estimate that a minimum of \$0.53/lge (liter gasoline equivalent) could be reached by 2020 in central producing hydrogen by water electrolysis or biomass gasification (U.S. DRIVE Partnership., 2013). Considering that the current US price of wholesale petroleum is \$0.35/liter, more efforts should be made to reduce hydrogen price and promote its use as energetic vector. Moreover, as each new clean and sustainable energy, hydrogen has to face scepticism and low acceptance from citizens that up to now are under-informed on the real necessity to change our consumption habits (Vassileva et al., 2016).

Nowadays, the development of hydrogen uses is mainly promoted by the automotive sector that is very active in the development of hydrogen-based vehicles. Since 1990s, some companies such as Toyota have focussed on building hydrogen vehicles working as close as possible to standard fuel car conditions. Toyota company works now with the Japanese government to imagine the hydrogen society of the future (Yoshida and Kojima, 2015). In this context, this company has even imagined to transform its production chain to use hydrogen as energy source with no carbon emission during all the car conception as shown in Figure 1.1-2. In this scheme, electricity produced by wind power plants is utilised to perform water electrolysis. The hydrogen generated is stored and compressed for further transportation and distribution in mobile hydrogen-fuelling stations. Toyota estimates to use this infrastructure by 2020 and become the first company using full hydrogen with neutral-carbon emissions (Pieraerts, 2016).

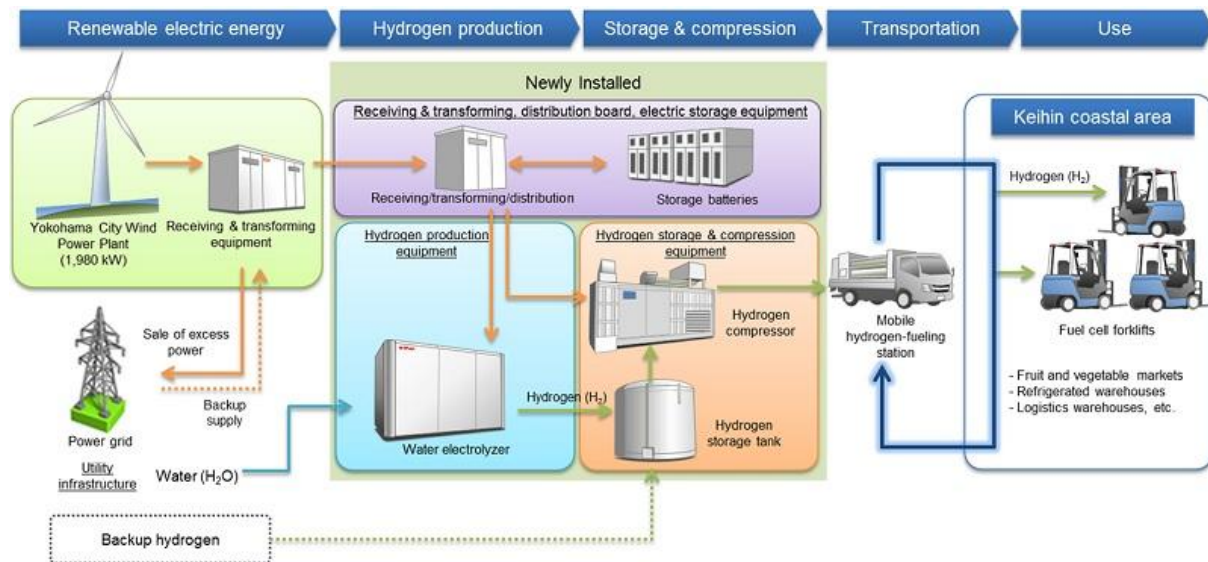


Figure 1.1-2: Toyota clean hydrogen production and supply chain (Kanagawa Prefectural Government et al., 2016)

Many other hydrogen plan projects on production, storage and distribution are funded around the world, mainly based on the development of hydrogen transportation. In USA, California is one of the most active States in terms of hydrogen infrastructure development. The Hydrogen Energy California project (HECA) was recently funded to develop hydrogen production through integrated gasification combined cycle, using petroleum (25%) and coal (75%) as feedstocks (U.S. Department of Energy, 2016). The aim of this project is to produce more than 400MWe a day with the capture of 90% of CO₂ emissions. In addition, the government invested \$50 million in hydrogen refuelling stations which should use 33% renewable hydrogen (California Energy Commission, 2014). Hence, 51 refuelling stations are planned to be activated by the end of 2016 and 90 stations by 2020. Hydrogen transportation is also completed by the development of a float of 26 fuel cell electric buses (FCEBs) owned by Californian bus companies like AC Transit, SunLine and Orange County.

In Europe, first Hydrogen projects were funded in the 80's by the European commission for application mainly in the aviation sector. Investment of €300 M accelerated hydrogen research in the early 2000's by funding 10 projects on hydrogen use and 6 on fuel cells (AFHYPAC, 2016a). Since 2015, the Hydrogen Mobility Europe project (H2ME) aims to join the European country efforts to develop the first European-network of hydrogen refuelling stations and fuel cell car fleet with a funding over €100 million.

Figure 1.1-3 presents the hydrogen refuelling stations (HRS) currently in operation in Germany, Scandinavia, France and United-Kingdom that were built during this project. Currently, 44 HRSs are

under operation. By 2023, 400 more HRSs are planned to be built in Germany, 150 HRSs in Scandinavia and 15-20 in France. UK plans to develop 65 HRSs by 2020 and 1,150 by 2030 (H2ME, 2015).

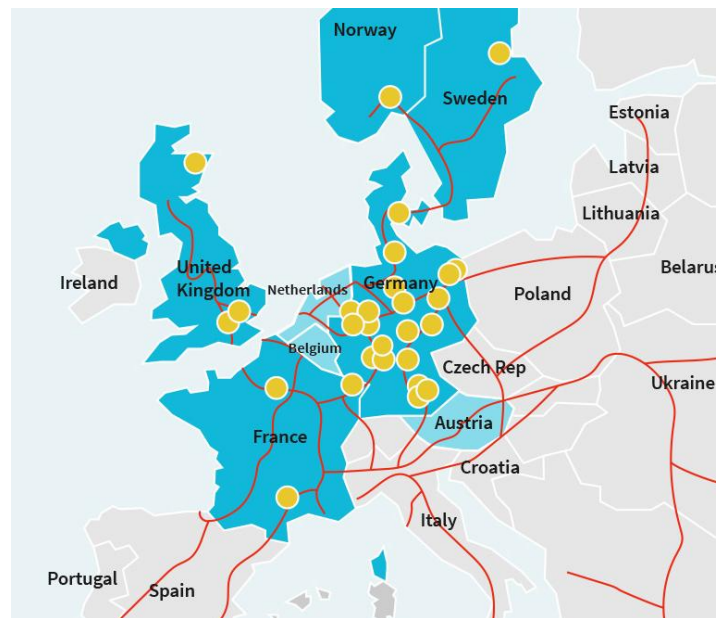


Figure 1.1-3: Countries taking part of H2ME project and infrastructures. Colours and symbols correspond to countries concerned by the project (■), observed countries (□), Refuelling stations (●) and main European roads (—). Source: H2ME roadmap (H2ME, 2015).

In France, the first commercial fleet of hydrogen vehicles was initiated in 2015 in Paris by the Parisian Society of Electric Taxi (STEP), with the purchase of five Hyundai ix35 equipped with hydrogen fuel cells. The project was supported by the Ile-de-France County, Mov'eo and Air Liquide companies and was associated with the building of one refuelling station. The STEP company estimates to increase their fleet to 70 vehicles by the end of 2016 (Poirot, 2015).

Other projects focused on the production and storage of hydrogen for infrastructure energy supply were also initiated. The HELION Company, a branch of the French company AREVA, has developed the *Greenergy BoxTM* (GeB) that produces hydrogen through water electrolysis, able to store the gas in an external reservoir (Verbecke and Vesly, 2013). In 2014, thank to 80% of public subvention, they installed their first GeB in a public building of the city of La Croix Valmer - Var (83), south of France - in order to answer to the high variation of energy demand between summer and winter (x10% during summer). Five other installations are planned to be built by 2018 (Viala, 2013).

Although the hydrogen energy sector is very active, if the 60 Mt of hydrogen currently produced per year would be only dedicated to energy usages, it would represent only 4% of the total

energy consumption (AFHYPAC, 2016b). Therefore, major challenges remain in improving the overall H₂ production by using clean and sustainable methods that can also outcompete petroleum and fossil fuels. The next chapter presents the different technologies available today to produce hydrogen.

1.2 Production of hydrogen

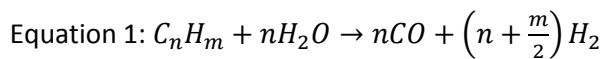
1.2.1 General technologies producing hydrogen

Today, large scale hydrogen production technologies are based on fossil fuel reforming which generate 96% of total hydrogen (Lin et al., 2012). Natural gas reforming and transformation of hydrocarbons for the production of hydrogen are advanced technologies that were developed on existing reforming petroleum platform. The remaining 4% of hydrogen are produced through water electrolysis, the most advanced renewable technologies that exist to produce the gas. But H₂ can also be obtained by organic biomass transformation, so called bio-hydrogen, through thermochemical and biological processes. For now, these bio-technologies remain at pilot scale demonstration but might be upscale in the next years. Table 1.2-1 resumes the main existing hydrogen production technologies and compares advantages and inconvenient of each one.

Table 1.2-1: techno-economic analysis of existing technologies to produce hydrogen. Information are extracted from (1) the US DRIVE Hydrogen Production Technical Team (HPTT) roadmap (2013), (2) Megret *et al.* (2015) and Parthasarathy *et al.* (2014)

	Method	Energy efficiency	H2 production cost	Production scale size/current trend	Major advantages	Major disadvantages
Fossil fuel reforming	Steam methane reforming (SMR) (natural gas reforming)	83%	\$0.55/lge (1)	Large/currently available	Most viable approach to begin building hydrogen market in near term; Lowest current cost; Existing feedstock & infrastructure	Dependence of fossil fuel; CO2 by-product,
	Coal gasification	46%	\$0.77/lge (1)	Large/currently available	Established; Cost-efficient; Provides low-cost synthetic fuel in addition to hydrogen	High reactor costs; Low system efficiency; Feedstock impurities; Need carbon capture and storage
Physico-chemical methods	Biomass gasification	46%	\$0.77/lge (1)	Mid-size/currently available	Renewable; Provides low-cost synthetic fuel in addition to hydrogen	High reactor costs; Low system efficiency; Feedstock impurities; Seasonal availability
	Bio-derived liquids reforming	>60%	\$1.56/lge (high temperature ethanol reforming) (1)	Large/currently available	Viable renewable hydrogen pathway in the near term; Existing infrastructure for some feedstocks	High costs (capital, operation and maintenance); Design for manufacturing; Feedstock quantity and quality required
	Electrolysis	67-74%	\$1.03/lge (if constant electricity price of \$0.049/kWh) (1)	Small/currently available	Proven technology, Emission free with renewable energy sources; Uses existing infrastructure; Uses fuel cell advances	Low system efficiency and high capital costs; Design for manufacturing
	Photo-electrochemical	4-18%	\$4.57-7.56/lge (1)	Under research	Operates at low temperatures; Clean and sustainable — using only water and solar energy	Need improving photocatalyst material; Low system efficiency; Cost-effective reactor; short-term technology; Large land area required
	Solar Thermo-chemical	<5%	\$3.91/lge (1)	Small scale/ in development	Emission free, renewable	High costs (capital, operation and maintenance); Large land area required; day/night dependence
Biological	Water biophotolysis	3%	\$0.32/lge (estimated) (2)	Under research	Clean and sustainable; Only need water and light; CO2 consumption	Relative high costs (capital, operation and maintenance); Low H2 production; Large land area required; Strong oxygen inhibition
	Photofermentation	4%	\$16-115/lge (2)	Under research – pilot scale	Clean and sustainable; Simultaneous treatment of effluent	High costs (capital, operation and maintenance); Low H2 production; Large land area required
	Dark fermentation	20%	\$2.64/lge (1)	Under research – pilot scale	Clean and sustainable, Simultaneous treatment of organic waste; No pure culture management	Expensive; Loss of H2 potential because of by-products accumulation; Sensitive to high H ₂ partial pressure; Long term instability
	Microbial electrolysis cell (MEC)	80%	\$1.47-8.84/lge (2)	Under research – pilot scale	Clean and sustainable, simultaneous treatment of effluent, High H ₂ production rate	Expensive; Scaled-up potentially difficult

During steam methane or oil reforming, the feedstock, i.e. methane or petroleum, first reacts with steam water to produce carbon monoxide and hydrogen (Brown et al., 2014). In the second reaction called water-gas-shift (WGS) reaction, carbon monoxide is converted into carbon dioxide and hydrogen. Conversion yields mainly depend on the H/C content as shown in Equation 1. H/C content is generally comprised between 3 and 5.



Hydrogen produced through this technology is very competitive to gasoline since costs are only related to production costs and not anymore to research and development (U.S. DRIVE Partnership., 2013). However, it remains a fossil energy –based technology with a high carbon impact, and that can only concern a near term use. Bio-derived liquid can be substituted to fossil feedstocks. These liquids, e.g. ethanol, glycerol, sorbitol, pyrolysis oils, glucose or methanol, are produced through biomass conversion (Piscina and Homs, 2008). Even though hydrogen production is more expensive than fossil fuels, it remains an accessible technology since infrastructures already exist for some residues (e.g. ethanol). Nevertheless, the costs of the bio-derived liquids directly impact the final hydrogen price.

The second most common technology is gasification which can be applied to both coal and biomass. Like steam reforming, feedstocks are submitted to high pressure and high temperature to break down molecules and produce carbon monoxide and hydrogen (Bhutto et al., 2013). Carbon monoxide is then transformed into carbon dioxide and hydrogen during the WGS reaction. Like steam reforming, this technology is also available and cost effective. However, the use of coal remains problematic because of high greenhouse gas (GHG) emissions and depletion of the reserves. That is why in the future, gasification will be preferentially performed on organic biomass, renewable feedstock leading to the production of hydrogen with zero CO₂ emissions (Bridgwater, 1995). The process can be performed on different types of biomass, such as straw, wood or algae (Parthasarathy and Narayanan, 2014). Biomass composition and its intrinsic characteristics such as particulate sizes can directly impact the resulting syngas composition (Kalinci et al., 2009; Vaezi et al., 2012).

The most common renewable way to produce hydrogen remains water splitting by electrolysis or thermal dissociation. Electrolysis consists in splitting water into hydrogen and oxygen by redox reaction (ROZENDAL et al., 2006). The chemical reaction is initiated using an electric current which

passes through two electrodes immersed in an electrolyte. Under a difference of potential of minimum 1.23V, water is split into oxygen and hydrogen. Both the price and the carbon impact of this technology directly depend on the mode of production of electricity. Indeed, electricity can be provided from nuclear energy or renewable energy e.g. solar and wind. According to the U.S. Drive Partnership report (2013), only nuclear, wind and solar energy are considered to be used in the long term because they are all considered as renewable and advanced technologies. Photoelectrochemical (PEC) hydrogen production is an example of technology which combines water electrolysis and solar energy: the electrochemical potential needed to split water is generated by sunlight exposure (Hisatomi et al., 2014). The major advantage of this technology is that it can be operated at low temperature (40-60°C).

Thermal water dissociation aims to irreversibly split water into H₂ and O₂ at very high temperature (3000 K). This process needs efficient heat source and resistant materials to work at such extreme conditions (Toklu et al., 2016). Currently, nuclear-based energy is the main heat source used, but solar thermochemical hydrogen production emerged in early 2000s (Perkins and Weimer, 2004). In this case, concentrated sunlight is used as power source to perform the redox reaction. However, this technology has many drawbacks since sunlight concentration process need to be greatly improved to be efficient, and materials currently used have to be replaced by more sustainable materials.

For now, fossil fuel reforming and water electrolysis are the only technologies economically viable. The US Department of Energy (DOE) estimates that processes with low or no greenhouse gases emissions will only be available by 2030 as shown in Figure 1.2-1 (U.S. DRIVE Patnership., 2013). Although biological processes are clean, sustainable, and can be used to manage organic waste and represent the most sustainable way to produce H₂, they still require a long term effort of Research and Development to be industrially viable. Biological hydrogen production methods are described in the next part.

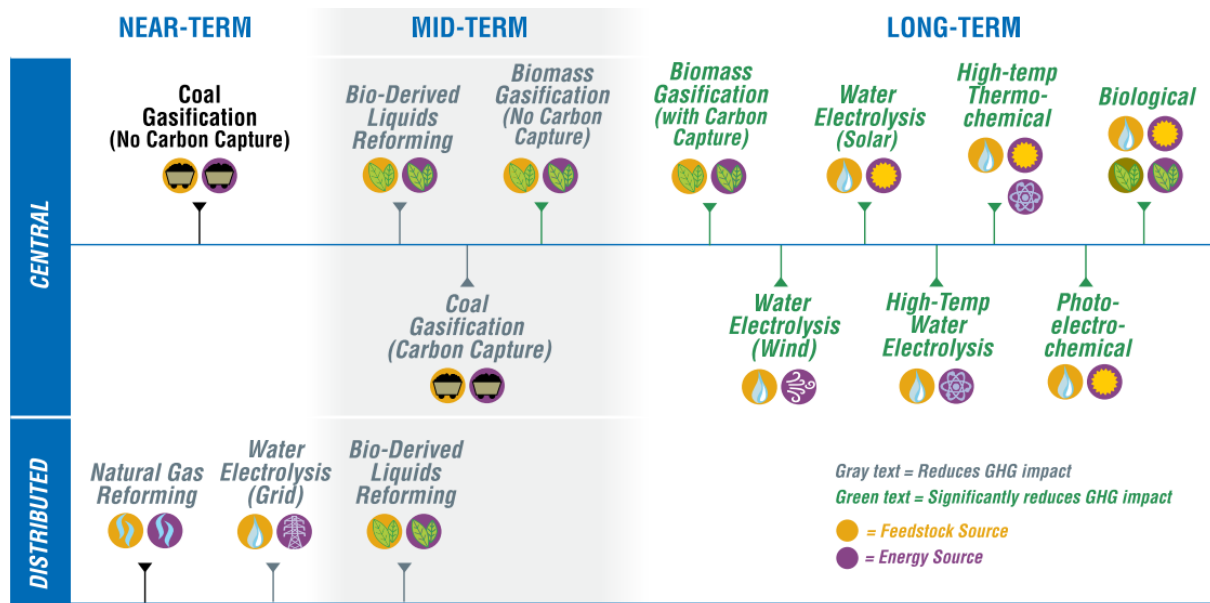


Figure 1.2-1: scenario of hydrogen producing method imagined by the U.S. Department of Energy (DOE) (FreedomCAR & Fuel Partnership, 2009)

1.2.2 Biological hydrogen production

Biological processes for the production of hydrogen are natural reactions developed for millenniums by microorganisms. In their cells, hydrogen plays a central role in energy production and conservation, but also in the maintenance within the cell of a constant oxydo-reduction potential and internal pH. Depending on the environmental conditions, microorganisms have developed metabolisms which can release hydrogen gas in their environment. Four main metabolic systems of biological hydrogen production exist: water bio-photolysis, photo-fermentation, dark fermentation and bio-electrochemical systems (Table 1.2-1).

1.2.2.1 Water bio-photolysis

Bio-photolysis is a method based on the principles of photosynthesis that requires light to split water into O_2 and H^+ . This process has been found in eukaryotic micro-algae and prokaryotic cyanobacteria (Yu and Takahashi, 2007).

Microalgae can produce hydrogen through direct bio-photolysis. In this case, photons with wavelength shorter than 680 nm are absorbed by the photosystem II (PSII) in the thylakoid compartment where water splitting occurs (Figure 1.2-2). The resulting proton accumulation forms a gradient which is used by the cell to generate energy under the form of ATP whereas electrons are transferred to the photosystem I (PSI) through an electron carrier. Photons under 700 nm

wavelength are absorbed by PSI and under certain conditions of starvation, hydrogen can be produced during the reduction of oxidised ferredoxin (Fd) by enzymes called hydrogenases. The overall direct biophotolysis reaction result in $2\text{H}_2\text{O} + \text{light} \rightarrow 2\text{H}_2 + \text{O}_2$ (Poudyal et al., 2015).

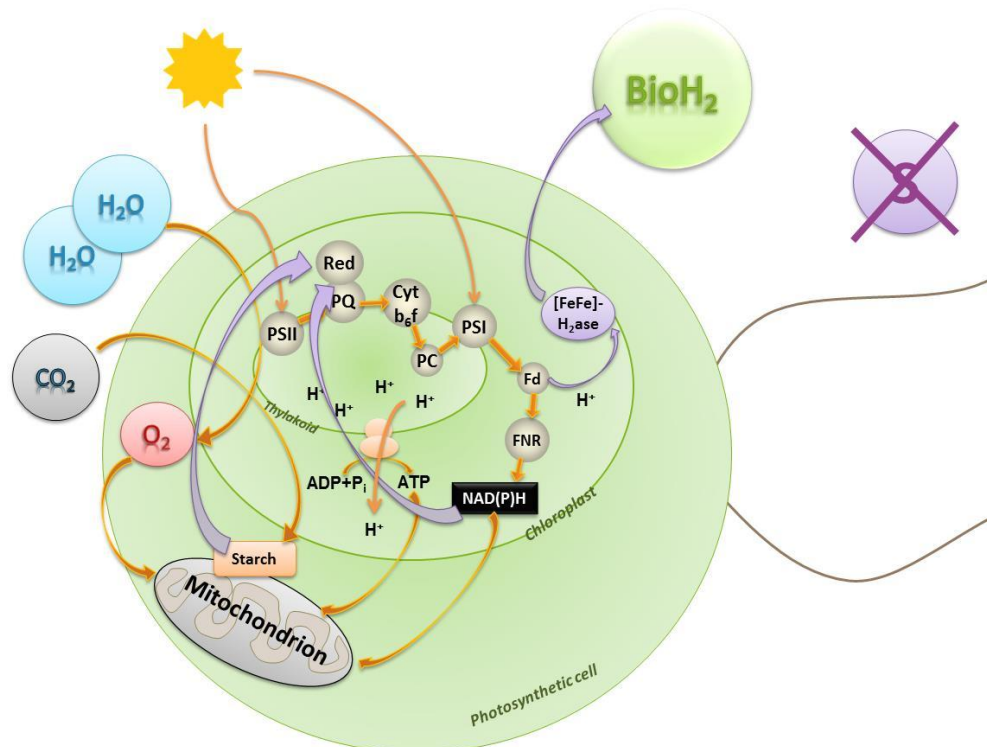


Figure 1.2-2: Example of direct bio-photolysis for the production of hydrogen in microalgae

This reaction can occur under very specific environmental conditions only, i.e. sulphur depletion and low oxygen partial pressure. However, sulphur and oxygen generation by photosynthesis are both required for cell growth. For that reason, two steps are required to produce hydrogen. During the first step, microalgae produce biomass and store reserves in a form of starch, in presence of oxygen and sulphur. During the second step, the production of hydrogen is catalysed by hydrogenase enzymes with no sulphur and low oxygen partial pressure to oxidise reduced ferredoxin and maintain the redox potential. The oxygen partial pressure is an important parameter for the production of hydrogen since hydrogenases of microalgae (based on iron core called [FeFe]-hydrogenase) are all inactivated at higher partial pressures than 2% v/v of oxygen (Yu and Takahashi, 2007). This high sensitivity for oxygen is the main limiting factor of hydrogen production. Very low hydrogen productivities of $0.07 \text{ mmol.h}^{-1}.\text{l}^{-1}$ are thus reported in the literature (MANISH and BANERJEE, 2008).

Oxygen sensitivity can be circumvented during indirect biophotolysis, generally performed by cyanobacteria. These microorganisms are aquatic photosynthetic bacteria able to produce hydrogen in two temporally or spatially separated steps. First, photosynthesis leads to water splitting and production of protons and carbohydrates through the activity of PSI and PSII photosystems. The cumulated carbohydrates are then consumed during night conditions, when no oxygen is produced by PSII, circumventing hydrogenase inhibition (Ananyev et al., 2012). The accumulation of protons and reduced ferredoxin during growth conduce to the production of hydrogen at this stage to maintain the oxydo-reduction potential of the cell.

The separation of oxygen/hydrogen reactions is also possible for bacteria that have nitrogen-fixing heterocyst compartments making the reactions physically separated. In this case, nitrogen-fixing cyanobacteria are able to grow in aerobic environment and concomitantly produce hydrogen with no oxygen inhibition (Shaishav et al., 2013). Hydrogen production in cyanobacteria is reported to be five times higher than those observed with microalgae, with $0.355 \text{ mmol.h}^{-1}.\text{l}^{-1}$ obtained from *Anabaena variabilis* (Rafrafi, 2012).

Overall, the main advantage of biophotolysis is the ability of bacteria to fix the atmospheric CO_2 and convert it into biomass (Table 1.2-1). Moreover, the process requires only water, nutrients and light to work. Algae are also rich in lipids and their biomass can be further valorised for the production of bio-diesel (Delrue et al., 2012). Some cyanobacteria such as *Spirulina platensis* are cultivated for their high nutritional value since they are rich in proteins, vitamins, minerals and essential fatty and amino acids (Belay et al., 1993).

However, because of the central role of light in the photosynthetic systems, photobioreactors have to be operated with a natural or artificial source of photons. At large scale, the use of natural sunlight remains the best option to minimize the operation costs. Nonetheless, large area is required to distribute photons to all photosynthetic cells, which contributes to increase the costs of this technology. The alternating day/night periods are also problematic for direct biophotolysis since the production of hydrogen is here not decoupled from photosynthesis and is lower at night (Androga et al., 2011). Moreover, light intensity depends greatly on climatic conditions, and because it is not constant throughout the year, meteorological conditions can also contribute to lower hydrogen production performances. The high oxygen sensitivity of the hydrogenases also leads to strictly control the operating conditions in reactors, which is a major technical challenge. To date, the production of hydrogen by bio-photolysis has only been performed at lab scale, but upscaling the

process could be a solution for both hydrogen production and carbon dioxide sequestration (Oey et al., 2016; Singh and Ahluwalia, 2013).

1.2.2.2 Photofermentation

Photofermentation is a biological system performed by purple non-sulphur (PNS) bacteria which also require light as energy source. When PNS bacteria are cultivated under anaerobic conditions, organic acids are oxidised leading to the accumulation of protons and electrons in the cell (Basak et al., 2014). In presence of light, photons at 750-900 nm of wavelength excite a transmembrane photosystem. That results to the transfer of electrons by several electron carriers up to the oxidation of ferredoxin (Figure 1.2-3). When the medium is depleted in nitrogen, i.e. high C/N ratio, the N₂-fixing enzyme, so called nitrogenase, is able to reduce the oxidised ferredoxin using the cell energy under the form of ATP (Adenosine Triphosphate) to produce hydrogen.

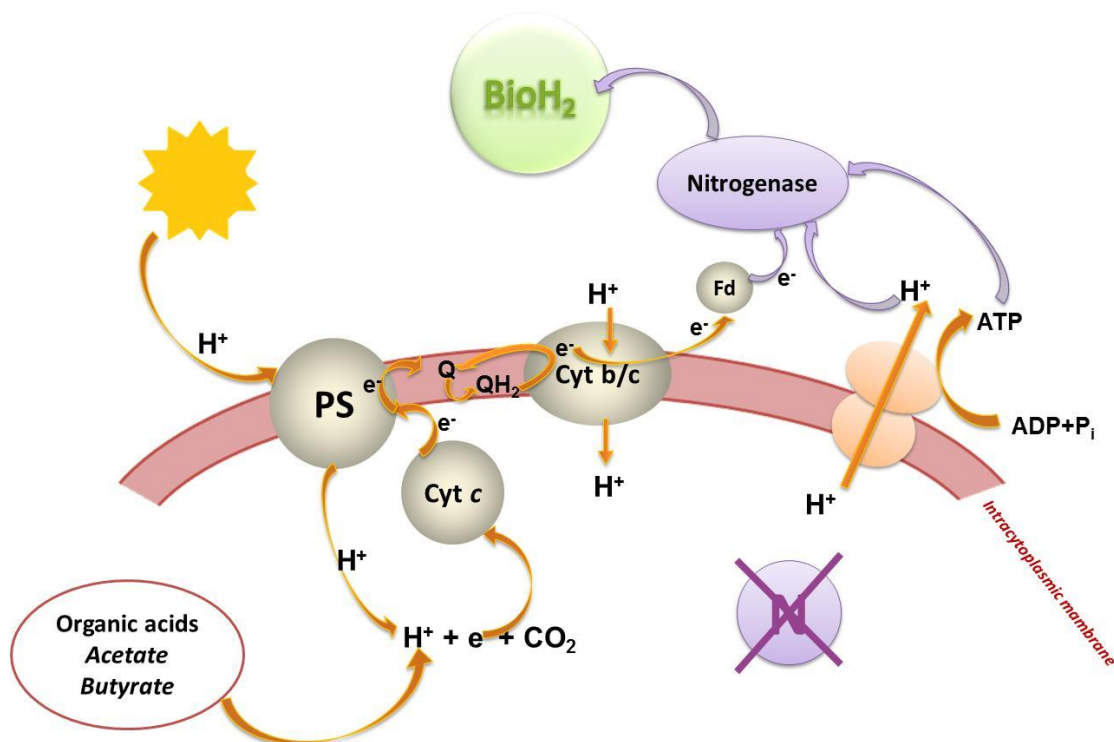


Figure 1.2-3: hydrogen production in purple non-sulphur bacteria, adapted from Deo *et al.* (2012)

Increase of light intensity leads to higher hydrogen production yield (Uyar et al., 2012). However, intensities higher than 400 W.m⁻² can also inhibit the production of hydrogen in most of PNS bacterial strains (Adessi and De Philippis, 2014; Kapdan and Kargi, 2006). In this system, not

oxygen but light can limit the gas production. Indeed, the presence of active hydrogen uptake enzymes contributes to decrease the overall hydrogen yield (Koku et al., 2002). To improve hydrogen production, research is now focusing on strain genetic improvement by deleting genes of the H₂-uptaking hydrogenases.

The main advantage of this process is the ability of PNS bacteria to grow on small carbon chain compounds allowing the use of cheap substrates such as wastewaters or outlet of previous degradation process such as dark fermentation (Uyar et al., 2012). Moreover, the photo-fermentative bacteria use a higher range of wavelength than photosynthetic microorganisms. The photofermentation productivities are thus higher than under biophotolysis, with around 150 mmol.h⁻¹.J⁻¹. But because the reaction has a high energy demand under the form of ATP, final energy yields are quite low, close to 4% only (Table 2.2-1, Megret et al., 2015).

Like photosynthetic systems, light is the central point for the production of hydrogen during photo-fermentation and the process is confronted to the same limits as direct bio-photolysis. Configuration of bioreactors is close to what has been developed for microalgae cultivation, but under anaerobic conditions (Adessi and De Philippis, 2014). Similarly, the use of solar light as source of photons can minimize the price of operation. Large-scale photo-bioreactors are generally put and operated in outdoor conditions. In addition, as photo fermentation works under anaerobic conditions, oxygen in bioreactor has to be purged. But oxygen removing through classical neutral gas replacement is difficult and expensive. Because PNS bacteria can fix CO₂ and atmospheric nitrogen, more expensive gas, e.g. argon, has to be used (Basak et al., 2014). The introduction of a new gas in reactor headspace means also that the final gas mixture has to be treated to obtain pure hydrogen, which contributes to increase the overall costs of this technology. Finally, upscaling bioreactors tends to lower the light conversion efficiency, to around 1%, than at laboratory scale at 4% (Deo et al., 2012).

1.2.2.3 Dark fermentation

Dark fermentation is different from the two previous processes because light does not act as a reactant of the system. The process derives from anaerobic digestion and is widespread in natural ecosystems (Dareioti et al., 2014). Indeed, fermentative bacteria can be found in various anaerobic environments, e.g. lakes sediments, manure, sewage sludge or digestive tracts. A group of microorganisms mainly composed of hydrolytic bacteria hydrolyse first the complex biomass and release simple molecules such as carbohydrates (Weiland, 2010). Resulting sugars are then

converted into hydrogen through the production of acetate and butyrate (Figure 1.2-4-green arrows; Latrille *et al.*, 2011). However, depending on bacterial metabolisms, the simple molecules can also be converted into other products without the production of hydrogen, such as lactate or solvents (Figure 1.2-4-orange arrows). In addition, when hydrogen-consuming bacteria are present in the medium, metabolites such as acetate or methane are produced by direct hydrogen consumption (Figure 1.2-4-red arrows).

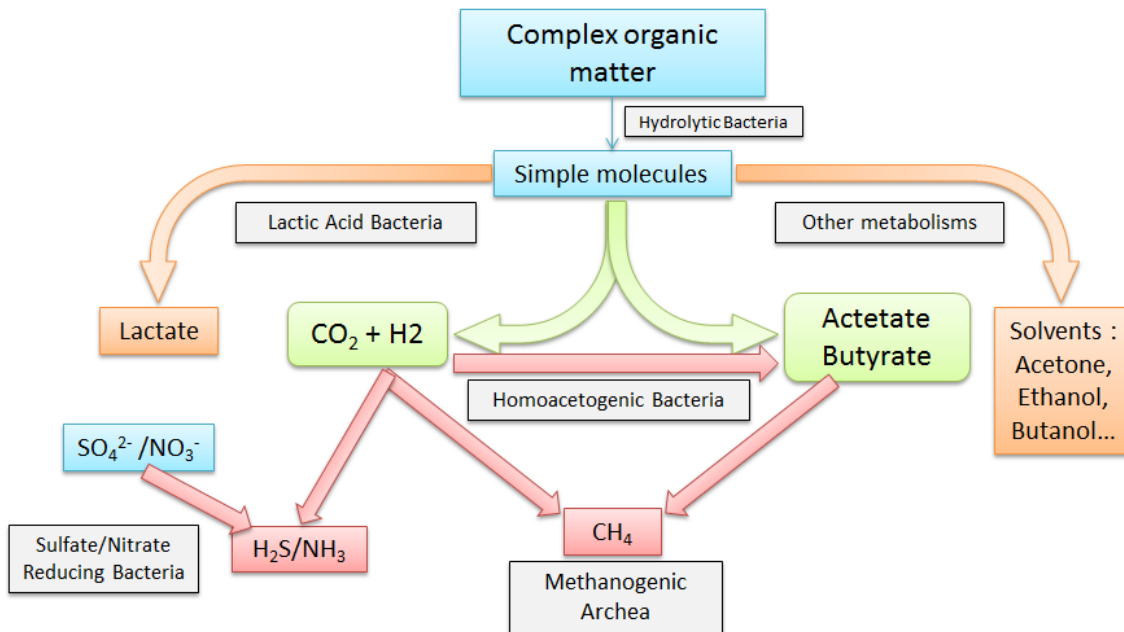


Figure 1.2-4: General scheme of dark fermentation, adapted from (Adapted from Latrille *et al.*, 2011)

The organic matter used as substrate can be from various origins. Hydrogen produced from crops from a culture only dedicated to energy production is called first generation hydrogen. However, such biomass enters in competition with food. However, the use of crops for energy production in substitution of food and cultivated on arable land presents severe ethic conflicts (FAO, 2011). The R&D efforts are therefore focussed on the production of hydrogen from second and third generation biomass, using organic waste and algae as feedstock for dark fermentation, respectively (Ghimire *et al.*, 2015; Lakaniemi *et al.*, 2013). These two types of biomass have complex structures which make their conversion into hydrogen more difficult than using simple sugars. Mixed bacterial cultures are generally preferred to the pure ones when using complex biomass since it increases the possibility to have bacteria efficient enough to convert this material (Kleerebezem and van Loosdrecht, 2007). In addition, they are mainly accessible to humans who can sample them directly in Nature, and no efforts of purity conservation have to be done during the process.

Hydrogen production yield by dark fermentation are higher than previous photo-bioprocesses, as presented before (Table 1.2-1). But substrate conversion rate into hydrogen remains low, not exceeding 20% of the stoichiometric yield, according to the cell biological activity. However, because the process is closed to anaerobic digestion, the technology developed for the production of methane can be adapted for the production of hydrogen, such as the UpFlow Anaerobic sludge Blanket (UASB) reactors after adaptation, accelerating thus the upscaling step. Productivities observed in continuous fermentation plant depend mainly on the type of sugar and associated reactor. Overall, productivities between $0.05 \text{ L}_{\text{H}_2} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ from agroindustry waste and $15.1 \text{ L}_{\text{H}_2} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ for glucose in Continuous Stirred-Tank Reactor (CSTR) working in mesophilic conditions are reported (Megret et al., 2015).

As the present thesis focusses on this process, dark fermentation principles are detailed in section 1.3.

1.2.2.4 Bio-electrochemical systems

A fourth system has been developed for the past decade called bioelectrochemical system (BES). Historically, BES is microbial fuel cells (MFC) originally designed for the production of electricity. But in the early 2000s', these systems evolved towards other uses and are more generally called MXCs, with X for the different types and application, i.e. desalination (MDC) or electrolysis (MEC) (Harnisch and Schröder, 2010; Moscoviz et al., 2016a).

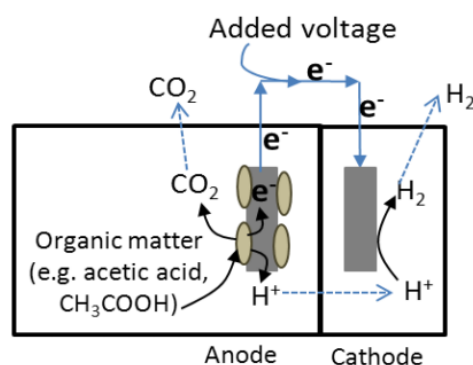


Figure 1.2-5: General scheme of microbial electrolysis cell (MEC). Figure from the U.S. DRIVE Partnership (2013)

For all MXCs, the anode is colonised by electroactive microorganisms which have the ability to consume organic acids and to transfer their exceeding electrons to the anode (Figure 1.2-5). The

migration of the electrons from the anode to the cathode generates an electric current which is utilised to perform specific reactions at the cathode, under a certain potential (Pant et al., 2012). Here, only 0.125 V of difference of potential is theoretically needed for the conversion of protons into hydrogen at the cathode, against 1.23V for abiotic water splitting in electrolyser (Hamelers et al., 2010; ROZENDAL et al., 2006). These processes present competitive yield since more than 80% of conversion yield were reported (U.S. DRIVE Patnership., 2013). Moreover, by using organic acids obtained by dark fermentation as substrate, the coupling dark fermentation-MEC could lead to yield close to 90% (Liu et al., 2012; Megret et al., 2015). However, the main drawback of the technology is the low courant density produced with a maximum of 100 A.m^{-2} against 1000 A.m^{-2} observed in classical electrolyser. Larger surface are thus required for MEC than in electrolyser to produce the same energy.

The BES technology is widely studied and available at laboratory scale and some pilot experiments have shown very promising results for wastewater treatment. As an illustration, a 1,000 L pilot-scale MEC was built to treat winery wastewaters and succeeded to remove 80% of the Chemical Oxygen Demand (COD) (Logan, 2010). However, the hydrogen produced was rapidly converted into methane and challenges remained in isolating the hydrogen produced at the cathode to avoid its reuse by methanogenic archaea (Cusick et al., 2011).

The gain of 10 folds energy to produce hydrogen makes the MEC a direct competitor of water electrolysis which only presents 67-74% of yield (Table 1.2-1). According to some reports this technology is near to be ready to produce clean industrial hydrogen (Logan, 2010; U.S. DRIVE Patnership., 2013). However, technological improvement needs to be performed in order to up scale the process and make it economically feasible (Clauwaert et al., 2008).

1.3 Dark fermentation

1.3.1 Principle of dark fermentation

During dark fermentation, bacterial activity converts organic residues into small organic acids, solvents and gas. Accumulation of reduced biochemical intermediates e.g. formate, nicotinamide adenine dinucleotide (NADH) and ferredoxin (FdH_2), can lead to the production of hydrogen to regulate the pH or maintain constant the internal redox potential (NADH and FdH_2) (Cai et al., 2011). Microorganisms with specific hydrogen-producing enzymes, so-called hydrogenases, have the ability

to release the excess in reduced elements through hydrogen gas by reducing two protons according to the following reaction: $2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{H}_2$.

Hydrogenases are metallo-enzymes which differ according to their metal content. The active site can be composed of only iron, so called [Fe]-hydrogenase or [Fe-Fe]-hydrogenase, or of iron, nickel and selenium, namely [Ni-Fe]-hydrogenase and [Ni-Fe-Se]-hydrogenase, respectively (Das et al., 2006). [Fe]-hydrogenases are found in archaea methanogens and cannot performed the redox reaction $2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{H}_2$. [Fe-Fe]-hydrogenases present a redox active iron site that makes possible this reaction (Kim and Kim, 2011). [Ni-Fe] and [Ni-Fe-Se]-hydrogenases cannot catalyse the production of hydrogen except some enzymes produced by PNS-bacteria (De Sá et al., 2011).

[Fe-Fe]-hydrogenases are generally involved in most of H_2 production processes. Their name comes from the presence of two iron nucleus in the catalytic subunit. [Fe-Fe]-hydrogenases have been found in most of the hydrogen-producing bacteria, e.g. clostridia, but also in hydrogen-consuming bacteria such as *Desulfovibrio vulgaris* since they are reversible enzymes. Most of the fermentative bacteria are able to produce many different types of hydrogenases allowing them to adapt quickly to environmental changes (Calusinska et al., 2010). Nevertheless, the catalytic subunit is generally encoded by a common gene called *hydA*, for which the variable regions of the gene sequence are differing from one bacterium to another. Quémeneur *et al* (2011a) used this property to differentiate H_2 -producing clostridial species within a microbial mixed culture after fermentation on different types of carbohydrates. They showed that the hydrogen-producing species affiliated to *Clostridium* genus were closely linked to the nature of the carbohydrate.

Hydrogenases are sensitive to some environmental conditions. [Fe-Fe]-hydrogenases are irreversibly inactivated by oxygen that destroys the active site of the enzyme (Stiebritz and Reiher, 2012). The inactivation occurs even in presence of traces of O_2 , imposing that the system has to be operated in strict anaerobic conditions. Moreover, hydrogenase can be inactivated in presence of hydrogen partial pressure higher than 0.3 atm (Esquivel-Elizondo et al., 2014). In order to circumvent such inhibition in bioreactors, the head-space can be purged with neutral gas such as nitrogen or carbon dioxide. By using CO_2 sparging during the continuous production of hydrogen, Kim *et al* (2006) succeeded to produce $1.68 \text{ mol}_{\text{H}_2}/\text{mol}_{\text{hexose}}^{-1}$ against $0.77 \text{ mol}_{\text{H}_2}/\text{mol}_{\text{hexose}}^{-1}$ without any flush.

As said before, hydrogen production occurs to maintain constant conditions within the microbial cell. During fermentation, the production of formate conduces to a decrease of pH. In order to keep it constant, enteric bacteria such as *Enterobacter* can convert the formate to produce

carbonate and hydrogen thanks to a formate lyase complex (Kim et al., 2010). Protons resulting from the conversion of formate by formate dehydrogenase are then reduced to H₂ by hydrogenase as presented in Figure 1.3-1-a (Moscoviz et al., 2016b; Tajima et al., 2015). In this case, hydrogen yield is limited to 2 moles of hydrogen produced per mole of glucose since two moles of formate can be obtained from hexose (Figure 1.3-2).

In strict anaerobes, hydrogen is also produced to maintain the redox potential through ferredoxin and NADH recycling (Pereira et al., 2011). Such process is more complex than the formate pathway found in enteric bacteria to maintain the intracellular pH. Indeed, the large diversity of enzymes offers many possibilities to maintain the redox potential as shown in Figure 1.3-1(b-e). According to Hallenbeck *et al.* (2012), hydrogenases can be NADH-dependent, Fd-dependent with ferredoxin reduction via NADH:ferredoxin oxidoreductase (NFOR), or bifurcating NADH-Fd-dependent. According to other studies, the reduction of ferredoxin occurs simultaneously with the conversion of NADH to NAD⁺ to produce the gas (Jang et al., 2013). At the contrary, other authors suggest that some hydrogenases can oxidize FdH₂ through the conversion of NAD⁺ to NADH (Liu et al., 2006). Saint-Amans *et al.* (2001) showed in *Clostridium butyricum* NADH accumulation through the re-oxidation of FdH₂ in presence of glycerol. This resulted in lowering the production of hydrogen compared to microbial culture growing only on glucose. In all cases, exceeding protons generated by oxidation reaction are then reduced to produce H₂.

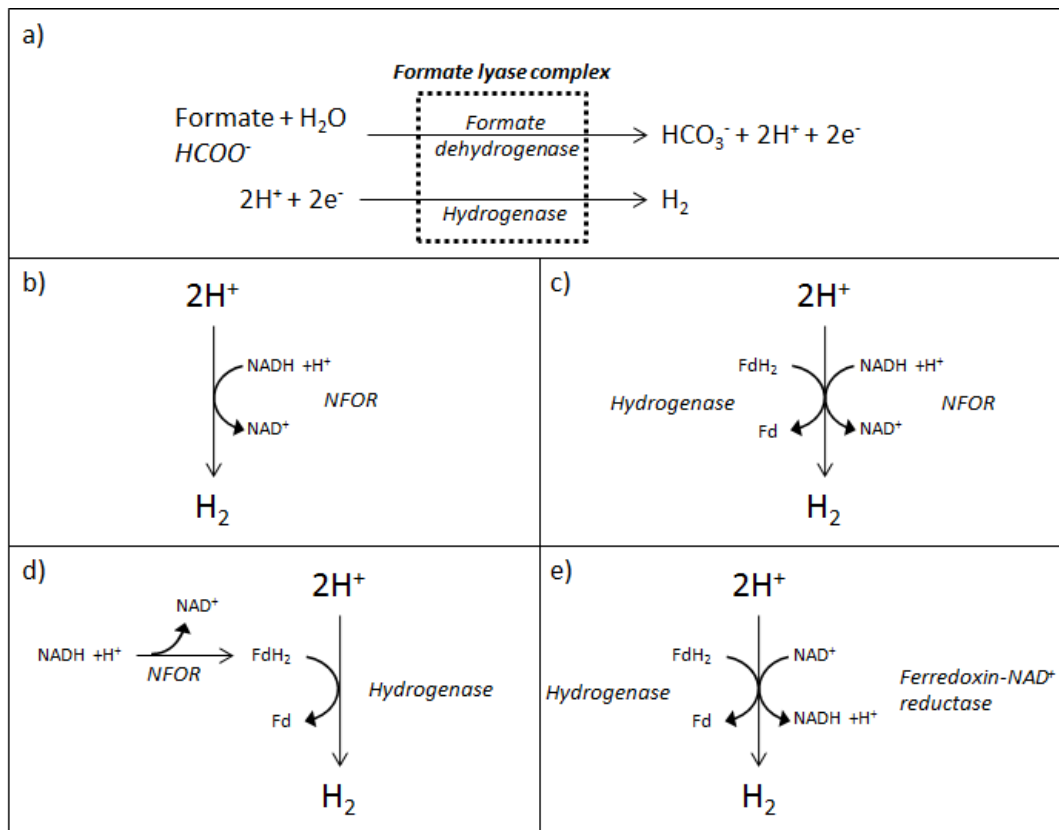


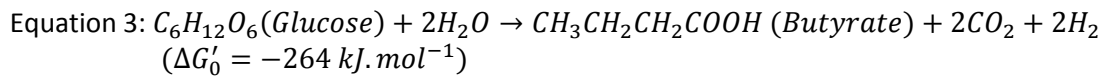
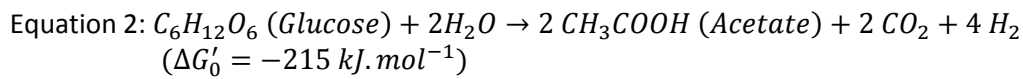
Figure 1.3-1: Enzymatic system to produce hydrogen developed by a) enteric bacteria from formate conversion, and by strict anaerobic bacteria from b) NADH-dependent hydrolase, c) bifurcating NADH-Fd dependent hydrolase, d) Fd-dependent hydrogenase with Fd reduction from NADH oxidation, and e) Fd-dependent hydrolase with production of NADH.

1.3.2 Metabolic pathways in dark fermentation

During dark fermentation, the accumulation of reduced intermediate products depends on final metabolic electron acceptor. As shown on Figure 1.3-2, pyruvate is produced during glucose degradation through the Embden-Meyerhof pathway and can be further converted into different metabolites, i.e. succinate, propionate, lactate, acetone, butanol, ethanol, formate, acetate and butyrate. Only the production of the last three metabolites can lead to the production of hydrogen, through formate splitting or accumulation of biochemical intermediates (FdH_2 and NADH) for acetate and butyrate as explain above.

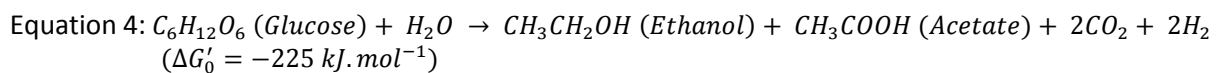
Acetate production is carried out by bacteria called acetogens. The production of this metabolite leads to the accumulation of 2 NADH and 2 FdH_2 , allowing to produce a maximum of 4 moles of hydrogen per mole of glucose consumed, according to Equation 2 (Ntaikou et al., 2010). Other fermentative bacteria accumulate two moles of FdH_2 through the butyrate-producing pathway

and the two moles of NADH produced during the Embden-Meyerhof pathway are recycled. Hence, a maximum of two moles of hydrogen can be produced as shown in Equation 3.



Generally, it was observed that the production of butyrate could not be dissociated to the production of acetate (Bastidas-Oyanedel, 2011). Butyrate production is initiated by the condensation of two acetyl-CoA to form butyryl-CoA which then reacts with acetate to produce one butyrate and recycle the acetyl-CoA (Figure 1.3-2-reaction ①). The production of butyrate appears after a first accumulation of acetate (Hoelzle et al., 2014). Therefore, it has been suggested that this pathway was a second option for the bacterial metabolism (Temudo et al., 2009). By producing simultaneously acetate and butyrate, a maximum hydrogen yield of $2.5 \text{ mol}_{H_2} \cdot \text{mol}^{-1}_{\text{glucose}}$ can be obtained, corresponding to a butyrate/acetate ratio of 1.5 (Hawkes et al., 2007).

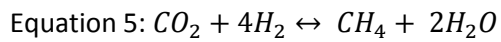
The production of the other fermentation products, i.e. ethanol, lactate, propionate, succinate, acetone and butanol, is possible with concomitant oxidation of NADH. Because the totality of the NADH produced is consumed during these metabolites formation, their presence in the medium is often associated to no hydrogen accumulation. Nevertheless, by association of a NADH-consuming metabolic pathway with one non NADH-consuming, hydrogen can be produced. It is the case of the co-production of acetate and ethanol which leads to the consumption of only two NADH if one molecule of acetate and one of ethanol are produced from two pyruvates. By this way, two moles of hydrogen are generated as showed in Equation 4 (Latrille et al., 2011).



In some cases, the production of metabolites results in hydrogen consumption like observed during methanogenesis and homoacetogenesis (Figure 1.3-2- red reactions).

Methanogenesis is realised by archaea methanogens present in anaerobic environment that can use hydrogen to produce methane through the reaction presented in Equation 5 (Costa and

Leigh, 2014). The sensitivity of archaea to acidity and heat allows developing pre-treatments to inhibit their activity. Generally heating at 90°C followed by ice cooling aims to select spore-forming hydrogen producer and inhibit methanogens (Kannaiah Goud et al., 2014; Venkata Mohan et al., 2008).



Homoacetogenesis is another hydrogen-consuming metabolic pathway but, contrary to methanogenesis, which is difficult to avoid. Homoacetogens are acetogenic bacteria that can switch their metabolic pathway to the consumption of hydrogen under stressful conditions. Therefore, acetogenesis/homoacetogenesis ratio does not necessarily depend on the bacteria phylogenetic affiliation but mainly on environmental conditions (Saady, 2013). The sole possibility to limit homoacetogenesis during dark fermentation is to stop the process just before the reuse of hydrogen. In the case of continuous reactors, it consists in reducing the hydraulic retention time (HRT) as shown by Si *et al.* (2015). The authors showed that a decrease of HRT from 24 h to 8 h resulted in an increase of the hydrogen yield from 1.25 to 1.47 mol.mol⁻¹_{glucose} thanks to a decline of the homoacetogenic activity. However, hydrogen-consuming microorganisms were not removed.

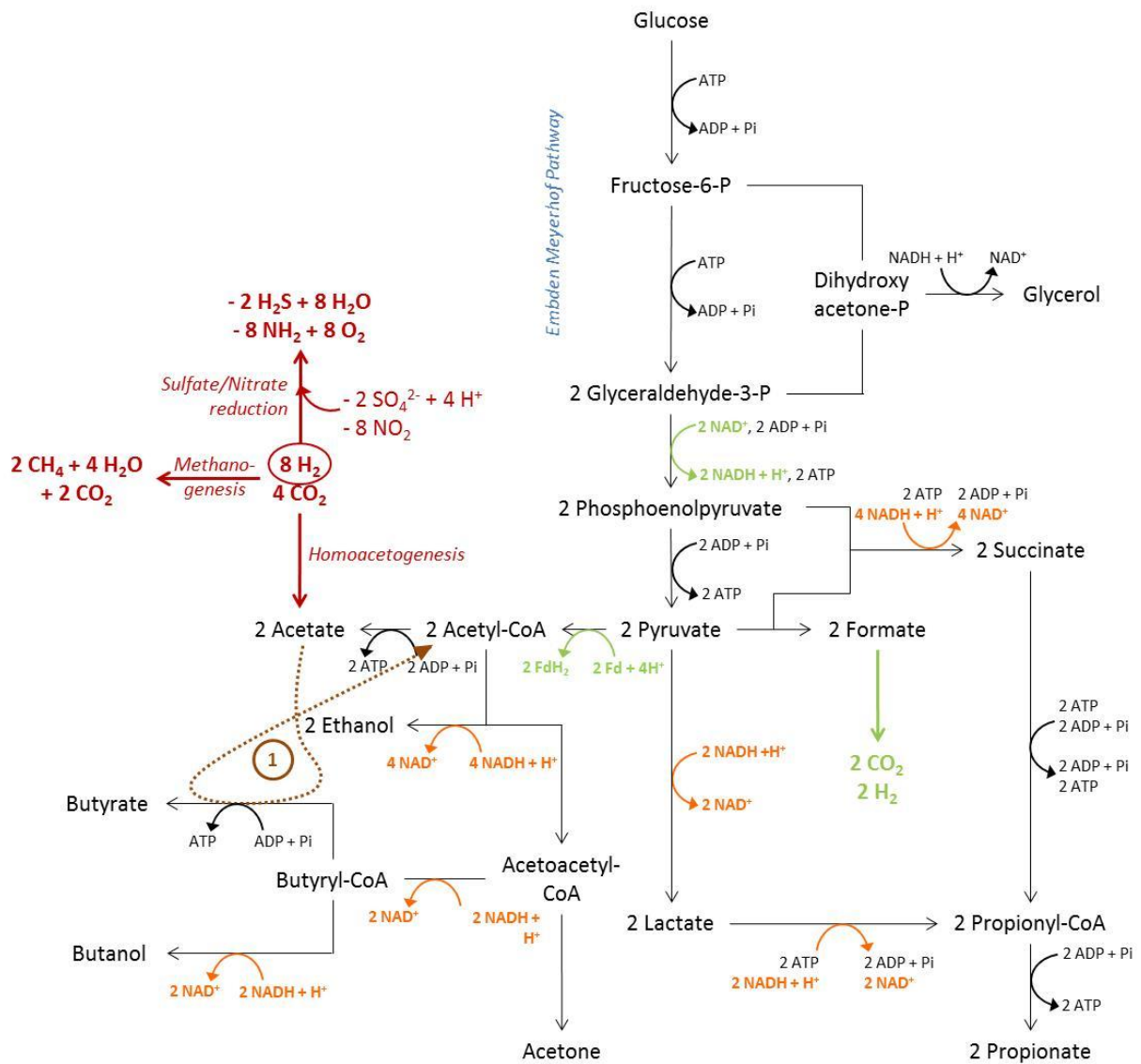
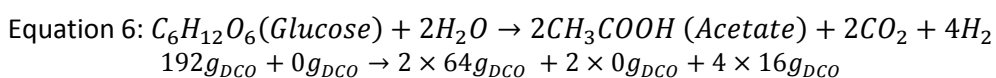


Figure 1.3-2: General and simplified fermentative metabolic pathway observed in anaerobic conditions from glucose. Green and orange arrows correspond to an accumulation and consumption of reduced intermediates, respectively, and the production of hydrogen through formate pathway. The red block corresponds to the principal pathway of direct hydrogen consumption. The reaction noted ① is the initiated butyrate production pathway by acetate consumption.

As presented in this section, the hydrogen produced during dark fermentation is always related to the production of by-products that accumulate in the liquid phase. Hence, hydrogen represents only a small fraction of the initial COD introduced into the system. By considering the production of four moles of hydrogen from one mole of glucose by the acetate-producing pathway, only 33% of the COD can be recovered under the form of H₂ as demonstrated in Equation 6.



Interestingly, the full conversion of glucose into hydrogen, resulting in $12 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$, was only obtained by *in vitro* enzymatic conversion of glucose. It could not be naturally obtained because of annex biologic activity of the cell (Woodward et al., 2000). Since hydrogen yield do not excess $2.5 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$ when using mixed cultures, 80% of the COD consisting in microbial biomass and metabolites must be valorised elsewhere to improve the process efficiency in terms of energy conversion (Megret et al., 2015).

1.3.3 Coupling dark fermentation with other biological processes to improve hydrogen yield.

Because of the microbial biology, dark fermentation accumulates mainly liquid metabolites, from 67 to 80% of the initial COD. In order to valorise these by-products, the process can be coupled to other biological methods as show on Figure 1.3-3. These couplings can increase the final biomass conversion into hydrogen when using bio-photolysis, bio-photofermentation and BES, or convert metabolites into valuable products such as biogas through anaerobic digestion, biofuel or bioplastics.

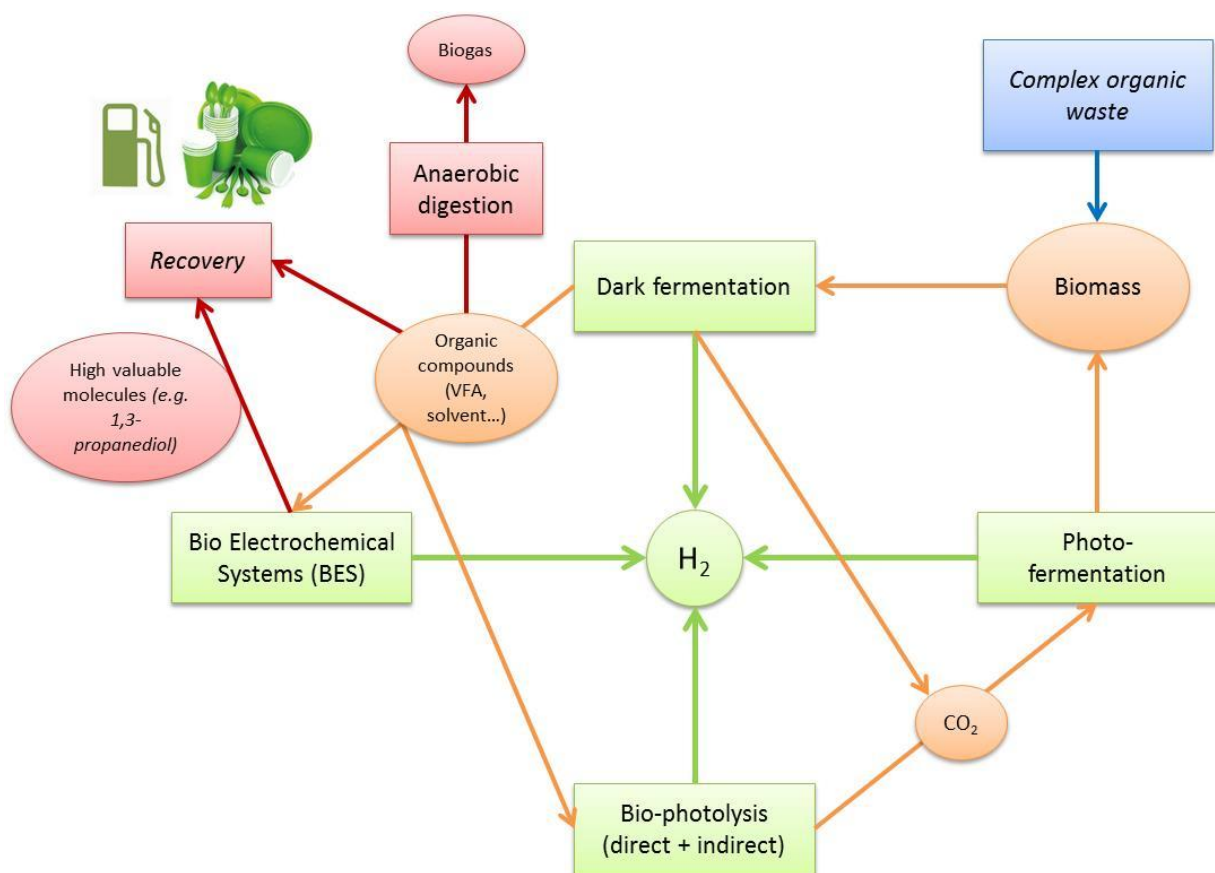


Figure 1.3-3: Coupling all biological hydrogen production systems to increase hydrogen yields.

Coupling dark fermentation with photofermentation has been studied in many types of reactors, e.g. batch, continuous and CSTR. Results in such two stage systems generally showed higher hydrogen yields than single stage dark fermentation, from 3.4 to 11.61 mol_{H₂}·mol⁻¹_{substrate} depending on the substrate and the inoculum used during dark fermentation (Azwar et al., 2014). Moreover, by using sequential DF/Photofermentation permit to treat effluent from dark fermentation by decreasing COD content during photo-fermentation. Through sequential DF/Photofermentation, Zong *et al* (2009) succeeded to remove 84.3% and 80.2% of COD from cassava and food waste feedstock, respectively.

The use of microbial electrochemical system on dark fermentation effluents also showed good results in terms of COD removing and hydrogen recovery. Using sludge with a VFA composition closed to the dark fermentation effluent, Liu *et al* (2012) succeeded to convert 90% of acetate and propionate into hydrogen. Even though BES requires energy, combination of these two technologies could provide high yield of H₂, up to 9 mol_{H₂}·mol⁻¹_{glucose}, with energy costs equivalent to the production of 1.2 mol_{H₂}·mol⁻¹_{glucose} (Liu et al., 2005).

In addition, fermentative process can be coupled to bio-photolysis system by injecting the carbon dioxide produced by fermentation into a microalgae photobioreactor. Although, to our knowledge, no work has been published to present the feasibility of this coupling, the use of photosynthetic microorganisms to fix CO₂ in a biorefinery context has been proposed as an efficient carbon capture technology (Pires et al., 2012). Dark fermentation effluent can also be valorised to produce microalgae biomass where lipids can accumulate and be further transformed into biodiesels. Indeed, in absence of light, some heterotrophic microalgae such as *Chlorella sorokiniana* can consume carbon sources such as acetate to produce biomass rich in lipids (Turon et al., 2016).

Anaerobic digestion is another bioprocess in which by-products of fermentation can be recycled in order to produce a bioenergy in form of methane. The gas mixture is composed of 5 to 20% of hydrogen in methane, forming a biogas called biohythane. This gas has a greater energy efficient than pure methane or natural gas. Moreover, its combustion limits the production of pollutants such as thin particles, formaldehyde or nitrogen oxide (NO_x) (Megret et al., 2015). Full scale two step biohythane production has already shown promising results. Hence, in Glamorgan University, a two-stage biohythane production pilot fed with sucrose led to a reduction of 93% of the initial COD with 1.62 mol_{H₂}·mol⁻¹_{hexose} accumulated during the dark fermentation process and 323 ml_{CH₄}·g⁻¹_{COD} during the anaerobic digestion step (Kyazze et al., 2007).

Coupling bioprocesses increases yields of substrate conversion into hydrogen or other energetic valuable products, e.g. lipids and methane. The combination of these systems has been proposed as economically favourable and could be very competitive with the other processes for hydrogen production. However, the price of hydrogen obtained using bio-processes mostly depends on the type of substrate used. By taking of organic waste as feedstock, it could be possible to make bio-processes more economically feasible.

1.3.4 Microbiology of dark fermentation

Depending on the type of bacteria used to perform dark fermentation, different metabolisms will be developed during the process, related to production of various amount of hydrogen. Fermentative bacteria can thus be related to hydrogen-producing, non-producing or consuming microorganisms.

1.3.4.1 Hydrogen-producing bacteria

Depending on culture condition, hydrogen-producing bacteria are generally affiliated to *Enterobacteriaceae* and *Clostridiaceae* for mesophilic bacteria, and to *Thermoanaerobacterium* for thermophilic strains (Elsharnouby et al., 2013).

Bacteria from the *Enterobacteriaceae* family are producing hydrogen through the formate pathway as explained in the previous section. Hydrogen-producing strains are generally affiliated to *Citrobacter* sp. *Enterobacter* sp. or *Klebsiella* sp. Studies on these bacteria showed hydrogen yields ranging from 0.04 to 0.83 mol_{H₂}·mol⁻¹_{added substrate} according to the strain (Patel et al., 2014). Because they are facultative anaerobes, *Enterobacteriaceae* bacteria are tolerant to oxidative stress which is an advantage in case of oxygen leakage in the reactor.

Well know, metabolic engineering can also be performed to improve the hydrogen production yield. Genetic modifications can be applied to enhance the hydrogenase activity by overproducing biochemical intermediates, e.g. NADH, or inactivating the production of undesired by-products such as lactate. Such mutation can improve hydrogen yields from 36% up to 149% (Song et al., 2016; Wang et al., 2013; Zhao et al., 2015).

Bacteria from the genus *Clostridium* produce hydrogen by re-oxidising their reduced biochemical intermediates through hydrogenases. Main strains identified are affiliated to e.g. *C. butyricum*, *C. pasteurianum*, *C. beijerinckii*, *C. acetobutylicum* or *C. bifermentans*. They are the main

hydrogen-producing species present in anaerobic reactors since their faculty to form spores allows them to resist to environmental stress and also because they have colonised most of natural environments. Because the oxidation of NADH and FDH2 leads to higher amount of hydrogen, clostridial species generally produce higher yields of hydrogen than *Enterobacteriaceae*, ranging from 1.61 to 2.36 mol_{H₂}·mol⁻¹_{glucose} (Hawkes et al., 2002). However, these amounts of hydrogen depend on culture conditions and carbon sources. Strains of *C. butyricum* exhibit H₂ yields between 0.6 and 2.2 mol_{H₂}·mol⁻¹_{hexose} from glucose at a usual pH between 5.2 and 6.5, while higher amount, 3.1 mol_{H₂}·mol⁻¹_{hexose}, can be obtained at pH 8 as observed by Junghare *et al* (2012). Using pentose-based substrates, same authors showed that lower hydrogen amount accumulated at the same pH since only 0.06 mol_{H₂}·mol⁻¹_{pentose} were obtained from arabinose and 0.59 mol_{H₂}·mol⁻¹_{pentose} from xylose. At the same time, strains of *C. beijerinckii* showed higher hydrogen yields on glucose with 2.81 mol_{H₂}·mol⁻¹_{glucose} (Lin et al., 2007).

In addition to the ability to produce hydrogen, many *Clostridium* sp. were identified to own efficient cellulolytic enzymatic material (Schwarz, 2001). Such species were identified in lignocellulosic environment such as rumen, manure or compost and are likely used to ferment complex organic biomass.

Like for *Enterobacteriaceae*, metabolic engineering is performed on pure strains to enhance hydrogen yield. Hence, mutation the *hydA* gene resulted in overexpression of [FeFe] hydrogenase expressed by *C. acetobutylicum* and by *C. tyrobutyricum* (Jo et al., 2010; Vonabendroth et al., 2008).

Mostly identified as hydrogen consumers, some bacteria affiliated to *Bacillus* genus can also produce hydrogen. Strains of *B. cereus*, *B. coagulans*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, *B. subtilis*, and *B. thuringiensis* were used to ferment diverse sugars and showed high hydrogen yields ranging between 0.23 and 5.6 mol_{H₂}·mol⁻¹_{substrate} depending on the carbon source (Kumar et al., 2013). Hence strain of *B. coagulans* sampled from anaerobic sewage sludge performed up to 2.28 mol_{H₂}·mol⁻¹_{glucose} and 5.6 mol_{H₂}·mol⁻¹_{cellobiose} (Kotay and Das, 2009, 2007).

The bacterial strains mentioned above were cultivated at mesophilic temperature but dark fermentation can also be performed under thermophilic (50-60°C) or hyperthermophilic (70-80°C) conditions. At these temperatures, strict thermophilic anaerobes producing thermostable enzymes are selected. They are mostly affiliated to *Thermoanaerobacterium* sp. which is phylogenetically close to *Clostridium* sp. It was observed that performing high temperature dark fermentation leads to higher yields of H₂, even on complex substrates such as cellulose (Gupta et al., 2015; Kargi et al.,

2012; H. S. Zheng et al., 2014). The use of temperature up to 50°C causes the inactivation of hydrogen-consuming bacteria. By studying dark fermentation on glucose at a range of temperature, 25-85°C, Jiang *et al* (2015) observed that 55°C was the optimal temperature for the production of hydrogen with 140 ml_{H₂}/g_{substrate added} against 15 ml_{H₂}/g_{substrate added} only at 35°C. Same improvement was obtained on cellulose with 110 ml_{H₂}/g_{substrate added}. In the case of complex substrates such as cellulose, improvement of hydrogen yield can be explained by the selection of thermophilic bacteria that produce thermostable lignocellulolytic enzymes. These enzymes have the capacity to keep their conformation and their activity at high temperature (Bhalla et al., 2013). They present faster enzymatic activity rate than those working under 50°C and provide thus a better potential of conversion. However, performing dark fermentation at so high temperature remains a technical challenge.

1.3.4.2 Characteristics of microbial ecology in mixed cultures used in dark fermentation

As said before, mesophilic inocula are composed of diverse hydrogen non-producing or consuming bacteria. Other *Clostridium sp.* can be linked to poor hydrogen accumulation by producing different end-products as final electron acceptor. Hence, *C. propionicum* and *C. homopropionicum* recycle NADH by producing propionate from lactate (Bundhoo and Mohee, 2016). Moreover, acetogenic *Clostridium sp.* can also produce acetate without hydrogen production through homoacetogenesis. In this case, bacteria produce acetate by consuming CO₂ and H₂ during autotrophic growth. Mixotrophic homoacetogens also exist and produce acetate by both consumption of organic substrates and hydrogen, which result in poor hydrogen yields (Bundhoo and Mohee, 2016; Diekert and Wohlfarth, 1994). As said before, the balance between acetogens and homoacetogens is often difficult to estimate since it depends on environmental conditions and not on bacteria phylogeny (Saady, 2013).

The presence of other anaerobes affiliated to *Bacilli* order is often associated to poor hydrogen production. They control their oxydo-redox potential by producing other reduced metabolites than hydrogen e.g. lactate or ethanol. Lactic acid bacteria (LAB) are common microorganisms responsible of the inhibition of hydrogen production during dark fermentation. They can be affiliated to seven different families, i.e. *Lactobacillaceae*, *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Leuconostocaceae*, *Streptococcaceae* and *Micorbacteriaceae* (Sikora et al., 2013). They are ubiquitous bacteria that act as competitors to the substrate since they consume sugars to produce lactate during homolactic fermentation, and lactate, ethanol and CO₂ during heterolactic

fermentation. They can also inhibit the production of hydrogen by secreting bacteriocins, toxins known to block the growth of other bacteria. Other strains of *Bacillaceae* than those presented in the previous section have been reported as lactate producers such as *Bacillus racemilacticus* (Hung et al., 2011).

In the case of mixed cultures, hydrogen producers and non-producers/consumers can cohabit. To overexpress the activity of hydrogen-producing bacteria, pretreatments based on the faculty of the clostridia to form vegetative spores can be applied to the inoculum. By pretreating an anaerobic inoculum at low pH, i.e. pH 2, using chloric acid, Lee *et al* (2009) showed an enhancement of the production of hydrogen of 3.2 times compared to the untreated culture. Heat or heat-shock (heat + ice cooling) treatment are generally used to select spore-forming bacteria since such high temperature is efficient to inactivate methanogenic archaea (Kannaiah Goud et al., 2014). By increasing the temperature of the pretreatment from 65 to 95°C, suppression of lactic acid bacteria from activated and anaerobic sludge was also observed (Baghchehsaraee et al., 2008). In this case, high temperature also selected more butyrate than acetate-producing bacteria which resulted in lower hydrogen, in accordance to the reaction stoichiometry. But heat pretreatments were found to be not efficient for long term experiment. By doing successive batch tests, Luo *et al* (2011) observed a progressive emergence of methanogenic and homoacetogenic activities under thermophilic conditions. Authors suggested that heat treatment does not kill but only slower hydrogen-consuming bacteria.

Optionally, co-culture of fermentative bacteria provides promising hydrogen yields. It was indeed observed that a co-culture of the facultative anaerobe *Enterobacter* sp. with a strict anaerobe *Clostridium* sp. allowed removing the dissolved oxygen initially present in the reactor and setup anoxic conditions. This would be a solution to pure culture cultivation of strict anaerobes in pilot plants that resulted in poor hydrogen yield because of oxygen inhibition (Hung et al., 2011; Patel et al., 2014). The use of these two bacteria also showed the possibility to combine two different biological activities to improve the conversion of complex substrate into hydrogen. By cultivating *C. acetobutylicum* with *Ethanoigenes harbinense*, Wang *et al* (2008) succeeded to produce $0.8 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose eq.}}^{-1}$ using microcrystalline cellulose as substrate. In this case, *C. acetobutylicum* played a cellulolytic role whereas the production of hydrogen was attributed to *E. harbinense*.

Other positive bacterial interaction was also observed between hydrogen producing and non-producing bacteria. During stress conditions, it was demonstrated that physical interaction permitted to two different bacteria specie to share cell material to subsist to nutrient depletion. During dark

fermentation of glucose by co-culture of *Desulfovibrio vulgaris* Hildenborough and *Clostridium acetobutylicum*, this phenomenon led to higher production of hydrogen than using *C. acetobutylicum* in pure culture (Benomar et al., 2015).

Such microbial association can be found in natural mixed communities in environment having poor oxygen concentration (Li and Fang, 2007). In addition to the benefits of the positive collaborations between microorganisms leading to higher hydrogen yields, mixed cultures represent an alternative to pure cultures especially when complex biomass like lignocellulose is used as substrate. Indeed, this type of biomass presents various residues polymerised to form a complex matrix (Singh and Wahid, 2015). Their biological degradation requires diversified enzymatic material to fully deconstruct the biomass. Such equipment cannot be found in totality in a single bacteria strain. The use of mixed culture leads to increase chances to develop efficient enzymatic material (Zuroff and Curtis, 2012).

The following Section 1.4 presents a state of the art of the main features of lignocellulosic biomass and their anaerobic degradation for the production of hydrogen.

1.4 Lignocellulosic residues as a resource for biological hydrogen production.

Intensified agricultural production has resulted on an increase of agricultural waste which have been evaluated to more than 220 billion tons per year (Ren et al., 2009). These quantities of organic matter overpass the capacity to Nature to recycle it, inducing environmental changes. Responsibility is now in managing waste emissions. Agricultural waste represents all residues obtained after various agricultural operations (Organisation for Economic co-operation and Development, 2001). It includes waste from farms, harvest waste but also fertilizers, pesticides, salt and silt drained by irrigation. The main proportion of agri-waste is organic and can be divided into two groups. Dry residues are straw, stalks and oilcakes, whereas wet residues are composed of manure slurry or grass silage (Megret et al., 2015). In all cases, lignocellulose remains the main component of these types of waste. Composition and biodegradation principle are developed in the next parts.

1.4.1 Composition of lignocellulose

Lignocellulose is the major organic carbon source on earth and the main constituent of plant cell wall. Its structure confers to the plant the rigidity and elasticity needed to grow and resist to

environmental attacks (Menon and Rao, 2012). Three polymers compose this complex matrix as shown on Figure 1.4-1.

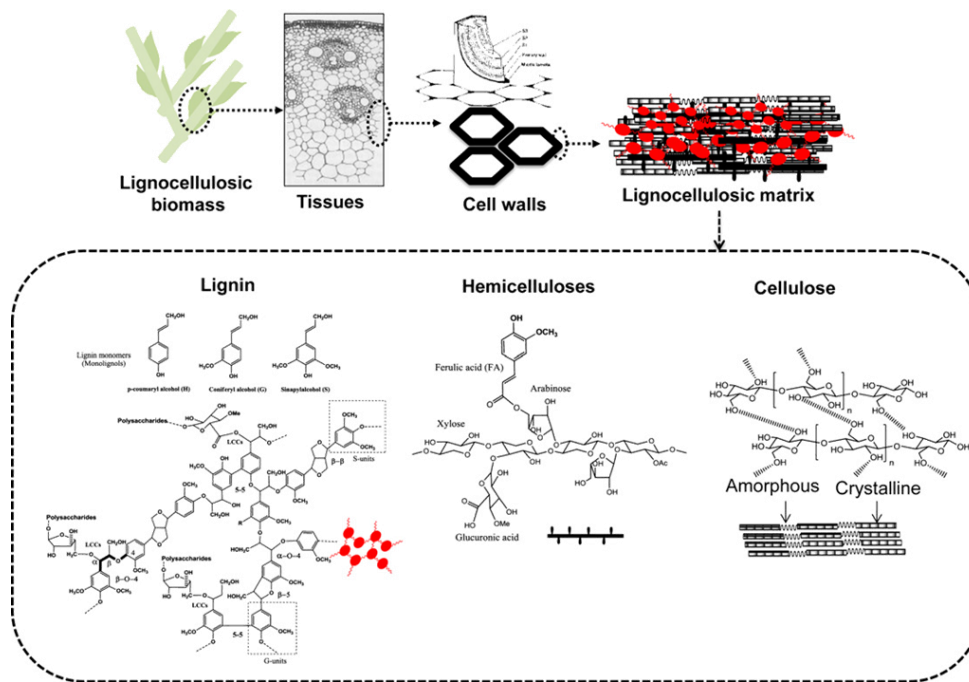


Figure 1.4-1: Structural composition of lignocellulosic biomass (from Barakat et al., 2013))

Lignin is a phenolic polymer which structure results from the oxidative coupling of three hydroxycinnamyl alcohols, namely *p*-coumaryl, coniferyl, and sinapyl alcohols (Wong, 2009). Once included within the lignin polymer, they are grouped in phenylpropanoid units and are named *p*-hydrophenyl (H), guaiacyl (G), and syringyl (S) units, respectively (Figure 1.4-2) (Pandey and Kim, 2011). The proportion of H, G and S subunits in lignin is different from one plant to another. Nevertheless, G and S-based lignin generally composes wood whereas herbaceous lignin plants have all the three units (Monlau et al., 2013a). Because of its phenolic composition, lignin is a natural barrier with a high resistance to microbial attack (Monlau et al., 2014). Indeed, only white and soft fungi are known to degrade lignin and almost no degradation of lignin is observed when using bacterial ecosystems, with some exceptions (Chanakya and Khuntia, 2014; Rouches et al., 2016).

Structurally, lignin is covalently linked to hemicelluloses to form lignin-carbohydrates complexes (LCCs) and surrounds hemicelluloses and cellulose, two polymers of carbohydrates (Monlau et al., 2013a).

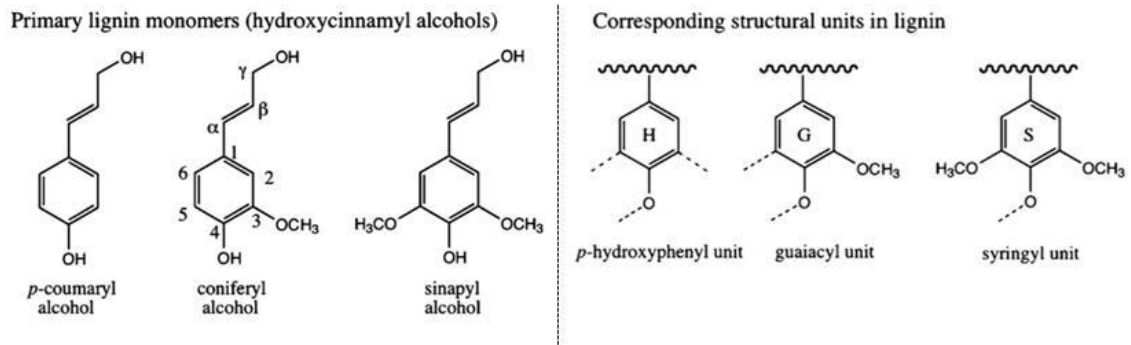


Figure 1.4-2: Primary lignin monomers and their corresponding form in lignin (from Wong, 2009)

Hemicelluloses are hetero-polymers of different carbohydrates which can be pentoses, hexoses, desoxyhexoses, uronic acids or amino sugars (Figure 1.4-3; Kögel-Knabner, 2002). Polymerisation of these molecules forms groups of polysaccharides which differ depending on the sugar composition (Scheller and Ulvskov, 2010). Xylans are the major group identified in lignocellulosic biomass, especially in straw. It is composed of D-xylose with $\beta(1-4)$ linkage. L-arabinose or 4-O-methyl-D-glucuronic acid can be branched in the second or third carbon position of the xylose (Gírio et al., 2010). It has been shown that the arabinose/xylose ratio is determinant for the biodegradability of hemicelluloses since arabinose is less biodegradable than xylose (Motte, 2013).

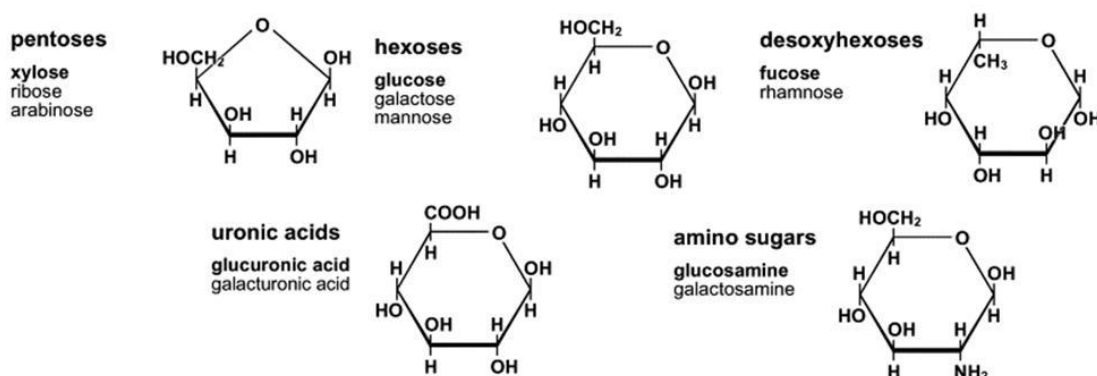


Figure 1.4-3: Main residues composing the hemicellulosic fraction (extracted from Kögel-Knabner, 2002)

Finally, hydrogen bonds link hemicelluloses to cellulose, the main component of plant cell walls. Cellulose is an homo-polymer of D-glucose with $\beta(1-4)$ linkages, where dimer of glucose forms the cellobiose, the primary pattern of cellulose (Estela and Luis, 2013). Cellulose in plant cells alternates two types of chain, i.e. the more biodegradable amorphous and less degradable crystalline

structures. Crystalline cellulose is composed of ordered cellulose chains packed so tightly that microorganisms and enzymes can hardly access them. Cellulose chains can be composed of up to 10,000 glucose units depending on the nature of the cellulosic biomass (Hallac and Ragauskas, 2011). Increasing the degree of polymerisation contributes to make even more difficult any microbial degradation as it will be presented in section 1.4.2.

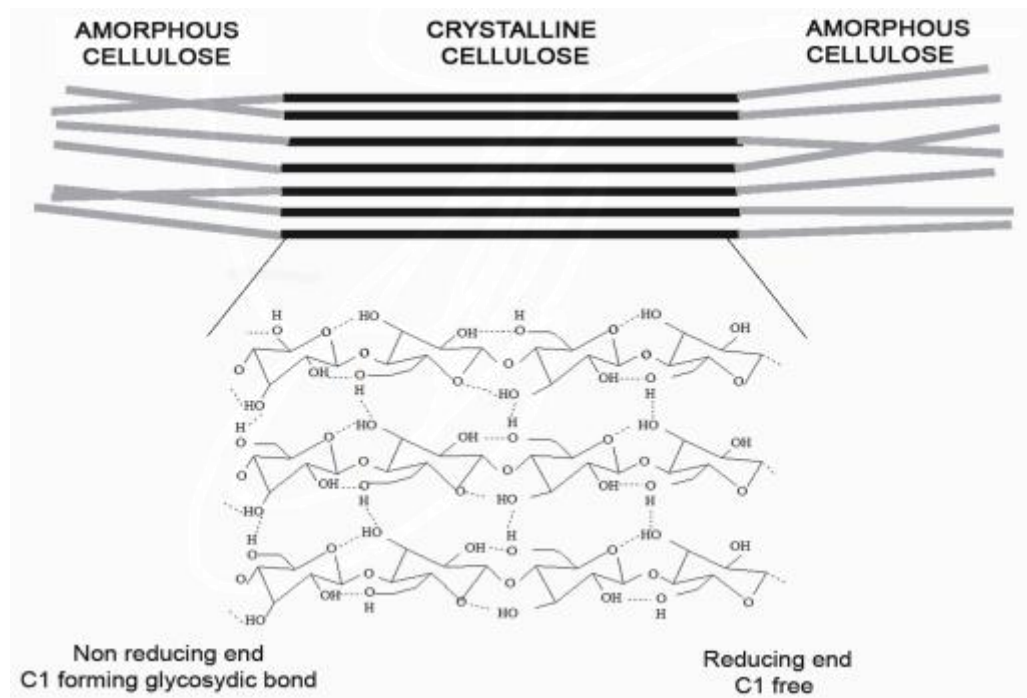


Figure 1.4-4: Chemical structure of cellulose (from Estela and Luis, 2013)

The proportion of each polymer in plant cell walls depends on plant nature and age, or even cell function. Vassilev *et al.* (2012) studied the proportion of the three components in different lignocellulosic biomasses. They observed that cellulose was the main polymer of most of the biomasses tested, whereas hemicelluloses were present in majority in wood, woody leaves, barks and twigs. Because it is composed of equivalent proportion of cellulose hemicelluloses and lignin, straw is generally used as lignocellulosic model (Motte, 2013).

1.4.2 Fermentative biological degradation

During dark fermentation, fermenting microorganisms can degrade lignocellulosic residues in anaerobic conditions. The first step of the process corresponds to biomass hydrolysis, where hydrolytic enzymes excreted by hydrolytic anaerobic microorganisms oxidise the glycoside bonds.

Carbohydrates are thus released in the medium under their monomeric form. The type of involved enzymes depends on the type of residues and monomeric linkage.

Cellulose is hydrolysed by three types of enzymes. The endo- β -1,4-glucanase breaks osidic linkages inside the chains of amorphous cellulose, the exo- β -1,4-cellobiohydrolases break linkages at the end of the chains and β -glucosidase breaks the released oligomers (Menon and Rao, 2012). In case of hemicelluloses, the diversity of residues composing the polymer implies the use different hydrolytic enzymes grouped under the name of hemicellulases, e.g. xylanases, mannanases, galactanases, arabinases (Saha, 2003). They release carbohydrates from the non-reducing extremities.

Degradation of lignin is carried out only under aerobic conditions by specific lignolytic microorganisms. Many works have focused on lignin degradation by soft mainly white-rot fungi but recent studies showed that bacteria also have the ability to assimilate this polymer by producing laccase and peroxidase (Bugg et al., 2011). Up to now, the sole bacteria identified with this faculty belong to the actinomycetes, α -proteobacteria and γ -proteobacteria classes. *Streptomyces viridosporus* cleaves β -aryl ether bound using extracellular peroxidase. Strains of *Pseudomonas*, *Nocardia* and *Rhodococcus* can also break down lignin, such as *Shingomonas paucimobilis*. Interestingly, DeAngelis et al. (2013) showed that *Enterobacter lignolyticus* could grow in anaerobic environment on lignin as sole carbon source. They found that the bacteria can convert lignin into monaryls, quinones and other aromatics that can then enter bacterial metabolism. Because lignin oxidation needs oxygen, no fermentative production of hydrogen from lignin has been reported so far.

Hydrolysis step required a lot of energy under the form of ATP and is consuming NADH (Figure 1.4-5). Hence, it is considered as the main limiting step for the production of hydrogen by dark fermentation (Guo et al., 2014). Hydrogen production mostly occurs during the conversion of soluble sugar released after hydrolysis. Carbohydrates that accumulate in the medium can then enter into the cells and be metabolized with potentially the production of hydrogen. Figure 1.4-5 shows the general metabolic pathways used to degrade different types of carbohydrates.

First, glucose follows the classical Embden-Meyerhof pathway to produce two moles of pyruvate, 2NADH and 2ATP (Lee et al., 2011). Pyruvate is then transformed depending on the metabolism developed by the microbial community as presented in part 1.3.2.

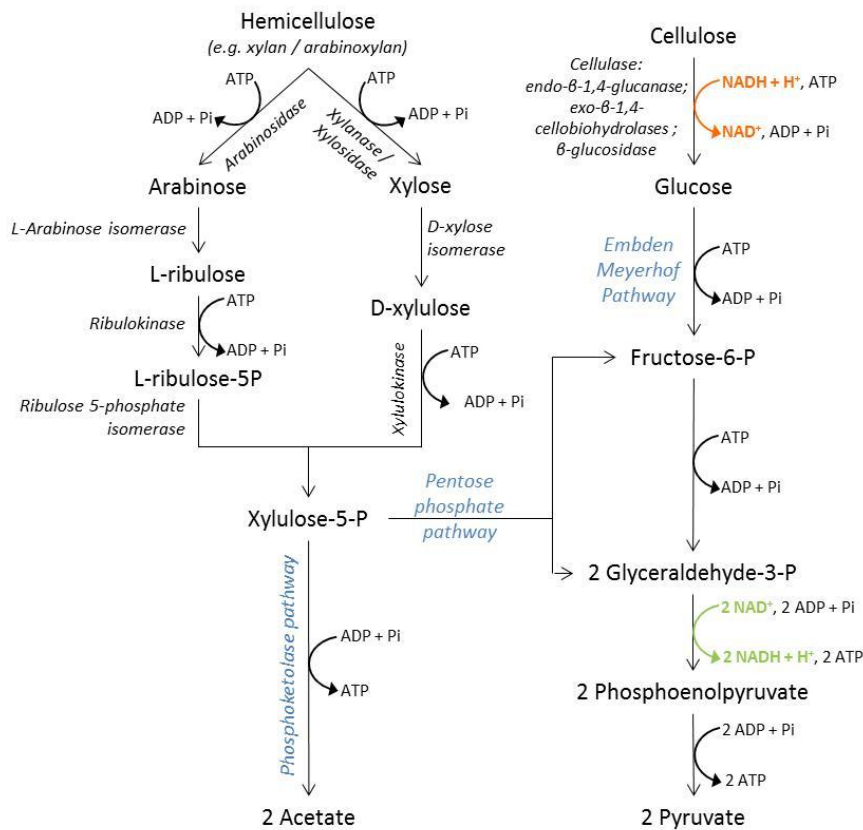


Figure 1.4-5: Metabolic pathway to convert hemicelluloses and cellulose through dark fermentation.

The integration of hemicellulose carbohydrates into cell metabolisms depends on the type of sugar. For more clarity and because they represent the major monomers of hemicelluloses, the following description is only focussed on xylose and arabinose integration into the bacterial metabolism. Once released in the medium, xylose and arabinose are converted into xylulose-5P by two different ways. Xylose is directly converted into D-xylulose and xylulose-5P by D-xylose isomerase and xylulokinase, respectively. In the case of arabinose assimilation, more complex enzymatic material is required. The sugar is first converted into L-ribulose and L-ribulose-5P before transformation into Xylulose-5P (Bettiga et al., 2009). Compared to xylose, the ability to convert arabinose seems to be less spread in microorganisms (Motte, 2013). The resulting xylulose-5P can join the common metabolic pathways through two different reactions. The first one is the phosphoketolase pathway where acetate is produced with accumulation of energy under the form of adenosine tri-phosphate (ATP). This pathway does not favour the production of hydrogen since no FdH₂ accumulates in the medium. However, hydrogen can be produced if xylulose-5P is converted into fructose-6P or Glyceraldehyde-3P through the pentose phosphate pathway (Skoog and Hahn-

Hägerdal, 1988). In this case, the xylulose-5P joins the last steps of Embden-Meyerhof pathway with the possibility to accumulate Fd_{red} and NADH.

Hence, the composition of the substrate directly impacts the biological process since ability to convert substrates is different. The next paragraph illustrates these influences.

1.4.3 Impact of lignocellulosic substrate on dark fermentation

Adapted from Chatellard, L., et al., 2017. Trends and Challenges in Biohydrogen Production from Agricultural Waste, in: Biohydrogen Production: Sustainability of Current Technology and Future Perspective. Springer India, New Delhi, pp. 69–95. doi:10.1007/978-81-322-3577-4_4.

1.4.3.1 The substrate composition conduce to different hydrogen production potential

Lignocellulosic biomass is promising candidate for hydrogen production by dark fermentation because of their high content in carbohydrates. As seen before, the quantity of hydrogen obtained from lignocellulosic materials depends on the metabolic pathway involved during the biological process. Because the metabolic pattern depends mainly on the type of carbohydrates, e.g. glucose, fructose, xylose or arabinose, the initial composition of lignocellulosic biomass impacts the final fermentative hydrogen potential. Overall, the polymeric structure will have a negative impact on hydrogen production since the hydrolysis step is energy and NADH consuming. Table 1.4-1 presents hydrogen potentials obtained from different lignocellulosic substrates.

Table 1.4-1: Literature review done on the production of hydrogen according different lignocellulosic substrates in batch reactor. The first block is pure commercial carbohydrate ranked by their degree of polymerisation. The second block is lignocellulosic biomass from waste or energy crops in alphabetic order. Hydrogen production is expressed in the following unit: a) $ml\ H_2 \cdot g_{TS}^{-1}$; b) $ml\ H_2 \cdot g_{VS}^{-1}$; c) $ml\ H_2 \cdot g_{TVS}^{-1}$; d) $ml\ H_2 \cdot g_{initial\ substrate}^{-1}$

Substrate	Type of inoculum	Process condition ($g \cdot l^{-1} / ^\circ C / pH$)	H ₂ production ($ml\ H_2 \cdot g^{-1}$)	References
Glucose	Wastewater treatment sludge	10/35/5.5	311 ^a	(De Sá et al., 2013)
Fructose	Wastewater treatment sludge	10/35/5.5	297 ^a	(De Sá et al., 2013)
Xylose	Buffalo dung compost	5/Room/-	340 ^a	(Prakasham et al., 2009)
Sucrose	anaerobically-digested sludge	10/37/5.5	125 ^a	(Quéméneur et

Substrate	Type of inoculum	Process condition (g.l ⁻¹ /°C/pH)	H ₂ production (ml H ₂ .g ⁻¹)	References
Maltose	anaerobically-digested sludge	10/37/5.5	117 ^a	al., 2011a) (Quéméneur et al., 2011a)
Cellobiose	anaerobically-digested sludge	10/37/5.5	117 ^a	(Quéméneur et al., 2011a)
Maltotriose	anaerobically-digested sludge	10/37/5.5	70 ^a	(Quéméneur et al., 2011a)
Beet pulp	Seed sludge	20/35/6.0	90.1 ^a	(Ozkan et al., 2011)
Grass silage	Mesophilic farm biogas digestate	-/35/6.2 -/70/6.0	11.5b 16.0b	(Pakarinen, 2008)
Grass	Cracked cereal	5 /35/7.0	4.39c	(Cui and Shen, 2012)
Leaf shape of mixed vegetable and potatoes	Indigenous microflora	-/37/6.7	19 ^b	(Marone et al., 2014)
Lettuce	Heat treated anaerobic sludge	20 /37/5.5	48 ^b	(Dong et al., 2009)
Perennial ryegrass (fresh)	Anaerobically digested sludge inoculum	20/35/5.2	21.8d	(Kyazze et al., 2008)
Perennial ryegrass (wilted)	Anaerobically digested sludge inoculum	20/35/5.2	75.6d	(Kyazze et al., 2008)
Potatoes	Heat treated anaerobic sludge	20/37/5.5	102 ^b	(Dong et al., 2009)
Reed canary grass	Sludge	-/35/-	1.3a	(Lakaniemi et al., 2011)
Rice straw	Anaerobic sludge	90/55/6.5	24.8 ^b	(Chen et al., 2012)
Soybean straw	Cracked cereal acclimated in Continuous Stirred-Tank Reactor (CSTR)	-/35/7.0	5.46 ^c	(Han et al., 2012)
Starch (cassava)	Cattle dung compost	18/37/6.8	199c.	(Zong et al., 2009)
Sunflower stalks	Anaerobic digested sludge	5/35/5.5	2.3 ^c	(Monlau et al., 2013b)
Sweet sorghum stalks	Anaerobic sludge	-/36/-	52.1e	(Shi et al., 2009)
Wheat bran	Activated sludge paper mill	100/36/7.0	50.6 ^d	(Pan et al., 2008)
Wheat bran	Digested sludge paper	100/36/7.0	28.6 ^d	(Pan et al., 2008)
Wheat bran	Mill corn stalk compost	100/36/7.0	22.6 ^d	(Pan et al., 2008)
Wheat bran	Wheat straw compost	100/36/7.0	17.7 ^d	(Pan et al., 2008)
Wheat stalks	Anaerobic digested activated sludge	60/35/6.5	23 ^c	(Chu et al., 2011)
Wheat stalks	Anaerobic digested dairy manure	60/35/6.5	37 ^c	(Chu et al., 2011)
Wheat straw	Seed sludge from H ₂ -producing	6/35/-	5.69 ^c	(Nasirian et al.,

Substrate	Type of inoculum	Process condition (g.l ⁻¹ /°C/pH)	H ₂ production (ml H ₂ .g ⁻¹)	References
	CSTR			2011)
Wheat straw	Mesophilic anaerobically-digested sludge	4/37/5.5	10.52 ^c	(Quéméneur et al., 2012a)
Wheat straw	<i>Clostridium butyricum</i>	40/35/7.2	9 ^e	(Li and Chen, 2007)
Wheat straw	Cow dung compost	25/36/7.0	0.5 ^d	(Fan et al., 2006)

When comparing studies on dark fermentation from pure carbohydrates, glucose is generally reported as the best candidate to produce hydrogen. Between 250 and 310 ml_{H₂}.g⁻¹_{glucose consumed} were collected from different works in batch experiments using mixed culture (De Sá et al., 2013; Prakasham et al., 2009; Quéméneur et al., 2011a). Similar production of hydrogen was observed from fructose fermentation with a maximum 297ml_{H₂}.g⁻¹_{fructose consumed} obtained by De Sá *et al* (2013). This production yield was accompanied by the production of butyrate and acetate representing 60 and 33% of all the metabolites produced, respectively. Traces of lactate and ethanol representing 7% of all metabolites seem to have a limited impact on H₂ production.

On xylose, the accumulated volume of hydrogen is mainly higher than glucose. Mäkinen *et al.* (2012) and Qiu *et al.* (2016) reported 121 and 211 ml_{H₂}.g⁻¹_{xylose consumed}, respectively, whereas De Sá *et al* (2013) and Prakasham *et al.* (2009) reached up to 321 and 340 ml_{H₂}.g⁻¹_{xylose consumed}. The volatile fatty acids represented 85%, 62 and 34% of all metabolites when 321, 211 and 121 ml_{H₂}.g⁻¹_{substrate consumed} accumulated, respectively. For the last case, accumulation of formate in the medium could explain the low production of hydrogen. Comparing all these studies, it can be concluded that higher hydrogen yields are obtained when proportion of butyrate increases. Moreover, studies on arabinose always showed lower hydrogen accumulation with a maximum of 104 ml_{H₂}.g⁻¹_{arabinose consumed} (Danko et al., 2008; Mäkinen et al., 2012). Formate was also identified in the final medium.

The impact of degree of polymerisation was studied by Quéméneur *et al.* (2011a) who showed lower hydrogen amounts after increasing the degree of polymerisation. More than twice less hydrogen was obtained from cellobiose, dimer of glucose with β-(1-4) linkage, than observed on glucose, with 111 ml_{H₂}.g⁻¹_{cellobiose consumed}. Similar hydrogen production was observed from maltose, dimer of glucose α-(1-4) branched, suggesting that the type of α or β linkage has no influence of hydrogen production. Using the glucose trimer maltotriose, with α-(1-4) linkage, only 70 ml_{H₂}.g⁻¹_{substrate consumed} accumulated. Such decrease of hydrogen amount was explained by a metabolic shift during the process. Hence, rising amount of ethanol and lactate was observed in correlation with an

increase of the degree of polymerisation. In Nature, glucose-based substrates present various degrees of polymerisation. Hydrogen yields close to what observed for glucose were obtained, probably due to the presence of directly accessible monomers. Hence, 199-240 ml $\text{H}_2 \cdot \text{g}^{-1}_{\text{substrate}}$ were reported from starch in batch reactors, and a maximum of 2.8 moles $\text{H}_2 \cdot \text{mol}^{-1}_{\text{glucose}}$ in continuous experiment (Zong *et al.* 2009; Su *et al.* 2009; Oztekin *et al.* 2008).

The negative influence of polymerised compounds has been reported in several studies as illustrated in Figure 1.4-6. It was shown that the prediction of hydrogen production can be done on soluble and easily accessible fraction of biomass, i.e. released oligosaccharides (Guo *et al.*, 2014; Monlau *et al.*, 2012). Hence it is not surprising that the maximum of hydrogen production obtained from lignocellulosic biomass is much lower. Using vegetable refuses, 48, 62, 71 and 102 ml $\text{H}_2 \cdot \text{g}^{-1}$ of TS were produced from lettuce, cabbage, carrot and potatoes, respectively (Dong *et al.*, 2009; Okamoto *et al.*, 2000). Potatoes are mainly composed of starch, while lettuce, closer to leaves, mostly contains lignocellulose with low lignin content. Hydrogen production yields were thus closely related to the component degradability. Same observation has been done by Venkata Mohan *et al.* (2009) who produced 124 versus 105 ml $\text{H}_2 \cdot \text{g}^{-1}$ of equivalent sugars from juice and pulp of mixed vegetable, respectively.

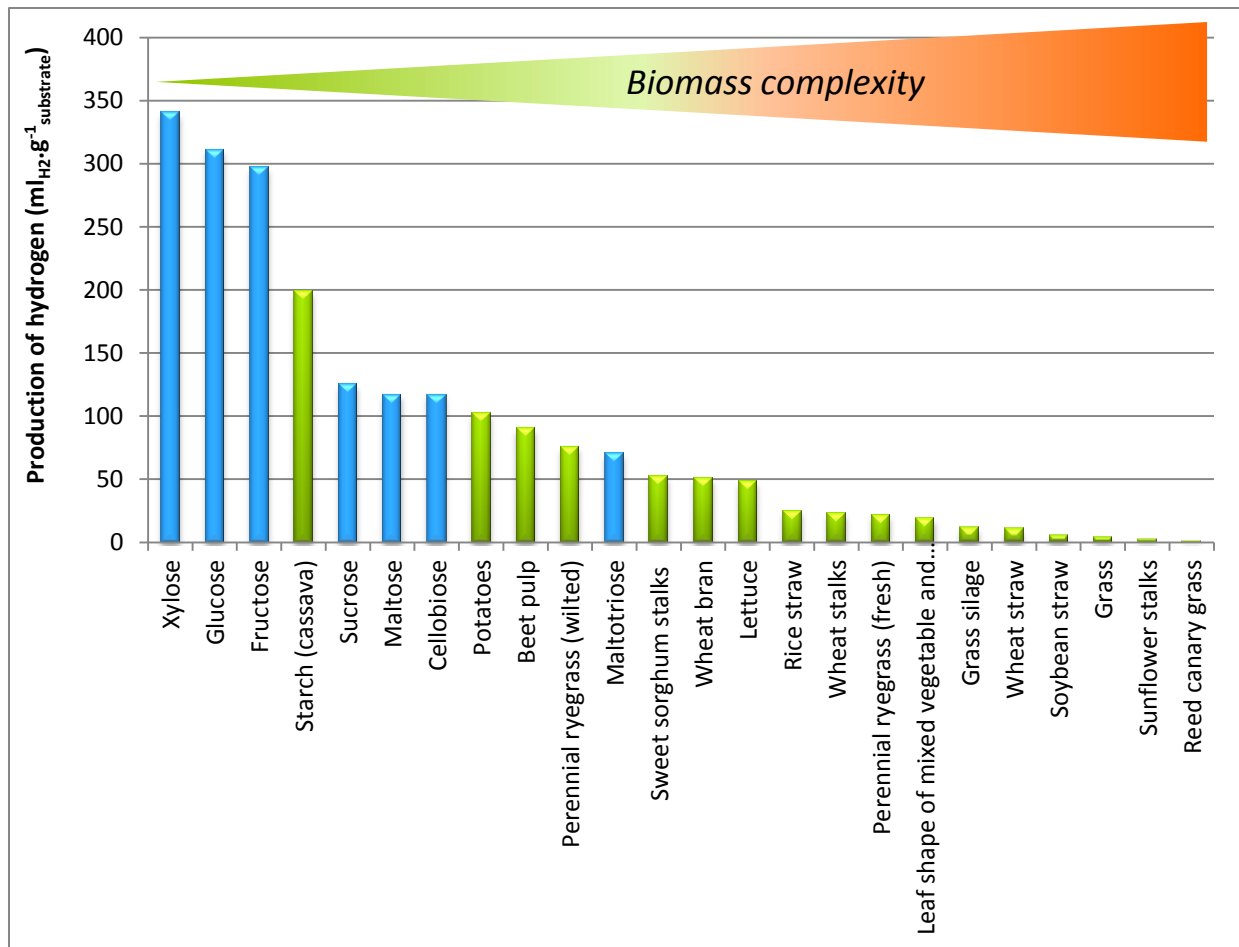


Figure 1.4-6: Production of hydrogen depending on the lignocellulosic substrate obtained in the literature. Blue bars represent pure commercial carbohydrates and green bars are natural lignocellulosic biomass.

Non-food crops residues are more resistant to biodegradation since lignocellulose with high lignin content represents the main component. Low biodegradable straws contain about 80% of lignocellulose (Guo et al., 2010), and hydrogen production are thus impacted. The fermentation of sorghum stalks, as an energy crop widely used for biofuel production, led to the production of 52 ml_{H2}·g⁻¹ TVS using mixed cultures in mesophilic conditions (Shi et al., 2009). Interestingly, hydrogen was only produced from the butyrate pathway since no acetate was identified. Lower yields were observed from other straws with yields ranging from 5 to 37 ml H₂·g⁻¹ of VS (Han et al., 2012; Li and Chen, 2007; Nasirian et al., 2011; Quéméneur et al., 2012a). In addition to low substrate conversion, propionate was produced and represented around 18% of all the metabolites. Same observation could be done on grass fermentation for which hydrogen yields ranged from 0.2 to 11.5 ml H₂·g⁻¹ VS (Cui and Shen, 2012; Lakaniemi et al., 2011; Pakarinen, 2008; Pakarinen et al., 2009). Acetate was the main metabolite, up to 64%, and propionate represented 7 to 25% of all by-products.

1.4.3.2 Use of pretreatment to increase hydrogen production yield

Because of its complex structure, the conversion of lignocellulose into valuable products is difficult. Therefore, lignocellulose pretreatments have been used to first deconstruct the material prior to microbial degradation and improve its conversion. At the beginning, these technics were developed for paper manufacturing to purify cellulose by removing lignin and hemicelluloses (Kleppe, 1970). Such methods are based on Kraft process and utilised sodium hydroxide and sodium sulphide to delignify the biomass. With second generation biofuels, the purified cellulose was valorised into ethanol by yeast fermentation, e.g. *Saccharomyces cerevisiae* (Karimi and Taherzadeh, 2007). However, liquid residues remaining after the Kraft process was rich in soluble sugars that compose hemicelluloses and could potentially be converted into ethanol. Over the past ten years, research focussed on the sugars purification from hemicelluloses to increase the amount of biofuels produced per initial quantity of lignocellulosic biomass (Talebnia et al., 2010).

In case of dark fermentation, high yields of hydrogen are obtained on soluble sugars from both cellulose and hemicelluloses fractions since the hydrolysis step is energy and NADH consuming (1.4.2). Lignocellulose pretreatments are generally selected according their faculty to hydrolyse the biomass. Performances of hydrogen production observed in the literature after pretreatment are presented in Table 1.4-2.

Table 1.4-2: Enhancement of biohydrogen production from different agricultural residues and operative conditions. Hydrogen production is expressed in the following unit: a) ml H₂·g_{COD}⁻¹ ; b) ml H₂·g_{VS}⁻¹ ; c) ml H₂·g_{TVS}⁻¹ ; d) ml H₂·g_{substrate}⁻¹. (* enzymes applied after alkali treatment: Cellulase, β-glucosidase and xylanase : 50, 25, and 50 units.g⁻¹ of TS)

Substrate / Substrate pretreatment	Type of inoculum / pre- treatment	Process conditions (g.l ⁻¹ /°C/pH)	H ₂ production (ml H ₂ ·g ⁻¹)	References
Barley straw/ 45min ozonation + enzyme hydrolysis	Cow manure and pond sediment	50/35/6.0	93.4 ^b	(Wu et al., 2013a)
Barley straw / Enzyme hydrolysis	Cow manure and pond sediment	50/35/6.0	35.05 ^b	(Wu et al., 2013a)
Beet pulp / 2M NaOH, pH=12 30min	Seed sludge	20/35°C/6.0	108.2 ^a	(Ozkan et al., 2011)
Beet pulp / 2M NaOH, pH=12 30min + microwave 700W 170°C 30min	Seed sludge	20/35°C/6.0	115.6 ^a	(Ozkan et al., 2011)
Beet pulp / 2M NaOH, pH=12 30min + 121°C 30 min	Seed sludge	20/35°C/6.0	66.7 ^a	(Ozkan et al., 2011)
Corn stover / 1.2% H ₂ SO ₄ + Steam explosion	Anaerobic digester sludge	27.5mM/35/5.5	3.21 mol H ₂ ·mol ⁻¹ gluc	(Datar et al., 2007)
Rice straw / Microwave heating 15 min at 140°C in 0.5% NaOH	Anaerobic activated sludge	60/35/6.5	150 ^c	(Cheng et al., 2011)
Soybean straw / 0.5 %NaOH 30 min	Cracked cereal acclimated in CSTR	-/35/7.0	10.47 ^b	(Han et al., 2012)
Soybean straw / 16 %H ₂ O ₂ 30min	Cracked cereal acclimated in CSTR	-/35/7.0	23.00 ^b	(Han et al., 2012)
Sunflower stalks / - 55°C 24h 4% NaOH +enzymes*	Anaerobic digested sludge	5/35/5.5	4.4 ^d	(Monlau et al., 2013b)
- 170°C 1h 4% NaOH +enzymes*			59.9 ^d	
			20.6 ^d	
			80.9 ^d	
Wheat straw / 2%H ₂ SO ₄ 120°C 90min	Seed sludge from H ₂ - producing CSTR	6/35/-	37.11 ^b	(Nasirian et al., 2011)
Wheat straw / 2%H ₂ SO ₄ 120°C 90min + cellulases, xylanases, and b-glucanases before	Seed sludge from H ₂ - producing CSTR	6/35/-	47.89 ^b	(Nasirian et al., 2011)

Substrate / Substrate pretreatment	Type of inoculum / pre- treatment	Process conditions (g.l ⁻¹ /°C/pH)	H ₂ production (ml H ₂ .g ⁻¹)	References
<i>fermentation</i> Wheat straw / 2%H ₂ SO ₄ 120°C 90min + cellulases, xylanases, and b-glucanases during <i>fermentation</i>	Seed sludge from H ₂ - producing CSTR	6/35/-	125.11 ^b	(Nasirian et al., 2011)
Wheat straw / Enzymatic cocktail (<i>Trichoderma</i> strain) during <i>fermentation</i>	Mesophilic anaerobically- digested sludge	4/37/5.5	21.61 ^b	(Quéméneur et al., 2012a)
Wheat straw / Sterilisation + enzymatic cocktail (<i>Trichoderma</i> strain) before <i>fermentation</i>	Mesophilic anaerobically- digested sludge	4/37/5.5	18.13 ^b	(Quéméneur et al., 2012a)
Wheat straw / Steam-explosion + enzymatic hydrolysis 25IU/g SECS	<i>Clostridium butyricum</i>	40/35/7.2	68 ^d	(Li and Chen, 2007)
Wheat straw / 2%HCl + 8min microwaves	Cow dung compost	25/36/7.0	68.1 ^c	(Fan et al., 2006)
Wheat straw / 45min ozonation + enzyme hydrolysis	Cow manure and pond sediment	50/35/6.0	87.35 ^b	(Wu et al., 2013b)
Soybean straw / 4 %HCl 30min	Cracked cereal acclimated in CSTR	-/35/7.0	20.71 ^b	(Han et al., 2012)

The best chemical process known to release soluble sugars uses acid solutions such as hydrochloric or sulphuric acids. Acids deconstruct lignocellulose by breaking the ether bonds that link lignin to carbohydrates (Alvira et al., 2010). Cellulose and hemicelluloses are solubilised whereas lignin precipitates at low pH. Concentrated acids present high hydrolytic efficiency but they are also toxic and corrosive, and can dramatically affect reactor operation (Monlau et al., 2013a). Dilute acids, i.e. 1-4% v/v acid, are generally used to circumvent this problem (Y. Zheng et al., 2014). With this approach, a 136 fold production of $68 \text{ ml H}_2\text{.g}^{-1}$ of TVS from wheat straw pre-treated with acid was observed, instead of the $0.5 \text{ ml H}_2\text{.g}^{-1}$ of TVS from straw by fermentation with a mesophilic cow dung compost (36°C) (Fan et al., 2006). This kind of treatment increases the overall biodegradability in high proportions as confirmed with soybean straws for which other authors reached 98.87% of degradation efficiency after fermentation by microorganisms issued from cracked cereals (Han et al., 2012). Acetate and butyrate were the two main metabolites produced during fermentation, with low amount of ethanol and propionate. In addition, a little decrease of propionate proportion was observed on pretreated soybean straw (Han et al., 2012).

To solubilise more biomass, acids can also be used in combination with other types of pre-treatments. Wheat straw grinding at 1 mm followed by 4% sulphuric acid pre-treatment during 90 min at 120°C led to a production of $37.11 \text{ ml H}_2\text{.g}^{-1}$ of VS (Nasirian et al., 2011). Combined with steam explosion, acid pre-treatment of corn stover led to an interesting hydrogen yield of $3 \text{ moles H}_2\text{.mol}^{-1}$ of sugars, with anaerobic digested sludge as inoculum (Datar et al., 2007). During the process of steam explosion, biomass is deconstructed by high temperature (hot water) and high pressure exposure. Here, water enters into the cell which starts to dissolve polymers, mainly hemicelluloses and cellulose, under the effect of heat. Then, a sudden decrease of the pressure makes the explosion of the biomass and leads to the reduction of particle size (Behera et al., 2014). The main drawback of these physico-chemical technics is that they are energy-consuming and these processes are considered as not economically feasible (Hendriks and Zeeman, 2009).

Acid pretreatment are usually carried out at very high temperature that can reach 160-220°C. In addition to contribute to the high price of the technology, it led to the production of inhibitory molecules such as 5-hydroxymethylfurfural (HMF) and furfural that inhibit microorganisms cell growth by breaking down DNA and RNA synthesis (Hu and Ragauskas, 2012; Jönsson et al., 2013; Monlau et al., 2014; Mosier et al., 2005). Other chemical can be utilised to limit the production of inhibitory compounds such as alkaline solution or ozone.

Alkaline solutions are selective for lignin since they break down the ester bonds between lignin and hemicelluloses through saponification reaction (Monlau et al., 2013a). Lignin removal leads to an increase of the cellulose and hemicelluloses bioaccessibility to enzymes. High degradability of 92.14% was observed after alkaline treatment with 4% NaOH on soybean straw (Han et al., 2012). However, alkaline pretreatments present low hydrolysis efficiency since long time of treatment is needed at ambient temperature. As an illustration, Playne *et al.* (1984) succeeded to improve the bagasse digestibility from 20 to 72%, using lime pretreatment during 192h. To decrease the process time, pretreatment can be performed at temperature up to 70°C, or in combination with microwave irradiation. Microwave disturbs the structure of the biomass by altering the structure of cellulose and partially removing lignin and hemicelluloses. The main advantage of this technics is the time process and energy input reduction comparing to other thermal methods (Cheng et al., 2011). Hence, association of microwaves and alkali severely improve hydrogen yields as has been observed on rice straw hydrolysate which reached a production of 155 ml H₂.g⁻¹ of TVS (Cheng et al., 2011). In this last example, acetate was the main metabolites identified after dark fermentation.

In the case of ozone, lignin and a part of hemicelluloses are solubilised by oxidation. Improvement of hydrogen production from 107% to 166% has been observed depending on the ozonation period (15-45 min) and on the type of substrate, i.e. wheat or barley straws (Wu et al., 2013a, 2013b).

However, alkaline solution and ozone, such as steam explosion, mainly deconstruct the biomass without hydrolysis of cellulose and sometimes hemicelluloses. Enzymatic hydrolysis often follows such pretreatment to release soluble sugars, i.e. cellulase and xylanase to hydrolyse cellulose and hemicellulose, respectively. In Quéméneur *et al.* (2012), an enzymatic cocktail secreted by an engineered *Trichoderma* strain, soft-fungi, was directly added to the fermentative process, leading to a two-fold increase in the hydrogen yield from wheat straw. The pretreatment also changed the acetate on butyrate ratio from 1.27 on raw substrate to 1.43 on the pretreatment one, close to the optimum of 1.5 (Latrille et al., 2011). However, precautions need to be taken when enzymatic pretreatment is separately carried out prior to fermentation. In this case the substrate should be sterilised to avoid direct sugar consumption by indigenous microflora (Quéméneur et al., 2012a).

By adding a concentrated solution of cellulase issued from *Penicillium decumbens* after steam explosion the hydrogen yield from corn straw was increased six fold, and reached 63 ml H₂.g⁻¹ of substrate with a pure culture of fermenting *Clostridium butyricum* (Li and Chen, 2007). The fermentation was also impacted since a shift from butyrate to acetate was observed. On barley

straw, the combination of enzymatic hydrolysis with a mixture of cellulase complex, endoxylanase and beta-glucosidase with 45min ozonation led to a production of 93.40 ml $\text{H}_2\cdot\text{g}^{-1}$ of TVS against 35.05 ml $\text{H}_2\cdot\text{g}^{-1}$ of TVS without ozone (Wu et al., 2013a). Following thermo-alkaline pre-treatment, enzymatic hydrolysis of sunflower stalks showed an increase of the hydrogen yields by a factor of 25 with 59.5 ml H_2/g of initial VS against 2.3 ml $\text{H}_2\cdot\text{g}^{-1}$ of initial VS with raw substrate (Monlau et al., 2013b). Enzymatic hydrolysis can also be used after dilute acid pre-treatment to increase soluble sugar. Finally, combining 2% sulfuric acid treatment with enzyme hydrolysis, cocktail of cellulases, xylanases and β -glucanases, Nasirian *et al.* (2011) reported a yield of 125.11 ml $\text{H}_2\cdot\text{g}^{-1}$ of VS, corresponding to an improvement of 3.5-fold the initial yield. However, composition of metabolites in these last studies was not discussed.

As showed in this section, biomass composition is a key factor for the production of hydrogen by dark fermentation. Even though pretreatments can increase hydrogen amount produced per unit of substrate, reported results can be different from one study to another. Such differences can be explained by the use of different bacterial communities. The next section will present the importance of microbial communities' composition on hydrogen-based metabolism.

1.5 Importance of microbial community on the dark fermentation processes in mixed culture

1.5.1 Evidence of the influence of inoculum origin on dark fermentation

As fermentative bacteria are spread into diverse natural ecosystems, many types of inoculum can be utilised to perform dark fermentation. Even using similar culture conditions, hydrogen potential can be strictly different during the conversion of same substrate. A summary of the hydrogen yields reported from various inocula fermenting different substrate is presented in Table 1.5-1. The table presents also the metabolic patterns and the main bacteria identified when molecular analysis was performed.

Using mesophilic conditions and pH 5.5-6, Quémeneur *et al.* (2012a) obtained 11 ml $\text{H}_2\cdot\text{g}^{-1}$ from wheat straw using a mesophilic anaerobically-digested sludge whereas only 0.5ml $\text{H}_2\cdot\text{g}^{-1}$ accumulated using cow dung compost (Fan et al., 2006). Same observation was made on wheat bran where 17.7; 22.6 and 17.7 ml $_{\text{H}_2}\cdot\text{g}_{\text{substrate}}$ with digested paper sludge, milk corn stalks compost and wheat straw compost as inoculum, respectively (Pan et al., 2008). Unfortunately, authors did not

describe the metabolites produced during the fermentation. Only Quémeneur *et al* (2012a) reported that butyrate was produced in higher amounts than acetate.

Consistently, the use of different inoculum origin on same substrate appears to influence the production of volatiles fatty acids (VFA) as demonstrated by Van Aarle *et al.* (2015) on carbohydrate-based biomass. Authors performed acidogenic fermentation, close to dark fermentation without the aim to produce hydrogen, using anaerobic sludge, granular sludge and acidogenic fermentation broth, separately. By fermenting wheat bran, anaerobic sludge produced acetate and butyrate in major proportion, representing 50 and 38% of the all VFA respectively, whereas propionate was a major component when using acidogenic fermentation broth and granular sludge. Moreover, acetate was not produced for the last inoculum. Such difference in metabolite profiles may impact hydrogen potential during dark fermentation.

Akutsu *et al* (Akutsu *et al.*, 2009a) were the first authors who evaluated the impact of the initial microbial community structure on hydrogen and metabolite production. They observed that clostridium-based communities were related to high hydrogen accumulation with butyrate as major metabolite, followed by acetate. At the opposite, co-culture of *Clostridium* sp. with *Bacteroidetes* sp. or *Bacillus* sp. bacteria resulted in lower hydrogen amount and metabolism driven to the production of ethanol and acetate. Similarly, Favaro *et al.* (2013) evaluated the influence of community structure changes by performing dark fermentation on organic waste with and without indigenous microorganisms and inoculum supplementation, i.e. heat pretreated granular sludge. They reported from 41.8 to 70.1 $\text{ml}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ depending on the microbial community, with the highest hydrogen amount observed using the indigenous microorganisms supplemented with granular sludge. This improvement of hydrogen production appeared to be due to an increase of the fermentation activity since the total quantity of metabolites increased without any metabolic shift.

Table 1.5-1: Results of hydrogen yield, metabolic patterns and identified bacteria obtained from dark fermentation performed on different inocula and substrates in the literature. Metabolites correspond to acetate (HAc); butyrate (HBut); caproate (HCap); ethanol (EtOH); lactate (HLac); propionate (HPr); formate (HFor); valerate (HVal) and succinate (HSuc). Hydrogen production are expressed as a) $\text{mol}_{\text{H}_2} \cdot \text{mol}^{-1}_{\text{substrate}}$; b) $\text{mmol}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{VS}}$; c) $\text{mmol}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{substrate}}$; d) $\text{mmol}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{TVS}}$; e) $\text{mmol}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{DCO}}$.

Inoculum	Substrate	H2	HAc	HBut	EtOH	HPr	HLac	HFor	HVal	HCap	HSuc	Major Bacteria	References
Anaerobically-digested sludge	fructose	1.84 ^a	34%	59%	6%	/	1%	/	/	0%	/	<i>C. sporogenes</i>	(Quéméneur et al., 2011a)
	glucose	1.79 ^a	33%	60%	6%	/	1%	/	/	0%	/	<i>C. sporogenes</i>	
	sucrose	1.67 ^a	86%	13%	1%	/	0%	/	/	0%	/	<i>C. sporogenes</i>	
	maltose	1.65 ^a	36%	58%	4%	/	0%	/	/	1%	/	<i>C. acetobutylicum</i>	
	cellobiose	1.56 ^a	35%	52%	9%	/	2%	/	/	1%	/	<i>C. cellulolyticum</i> ; <i>C. sporogenes</i> ; <i>C. Saccharobutylicum</i> ; <i>C. kluyveri</i> ; <i>C. acetobutylicum</i>	
Wastewater treatment sludge	maltotriose	1.38 ^a	31%	48%	17%	/	3%	/	/	1%	/	<i>C. acetobutylicum</i>	
	fructose	2.09 ^a	13%	87%	/	/	/	/	/	/	/	/	(De Sá et al., 2013)
	glucose	2.19 ^a	16%	84%	/	/	/	/	/	/	/	/	
xylose	1.88 ^a	15%	85%	/	/	/	/	/	/	/	/		
Hot spring culture	xylose	0.7 ^a	25%	34%	5%	0%	1%	34%	/	/	/	<i>C. acetobutylicum</i> ; <i>C. tyrobutyricum</i>	(Mäkinen et al., 2012)
	arabinose	0.42 ^a	23%	31%	13%	0%	8%	25%	/	/	/	/	
Seed sludge	xylose	2.24 ^a	17%	62%	13%	8%	/	/	/	/	/	<i>C. saccharobutylicum</i> ; <i>Clostridiales sp</i>	(Qiu et al., 2016)
Anaerobic activated sludge	hydrolysed starch	2 ^a	18%	81%	1%	0%	/	/	/	1%	/	/	(Su et al., 2009)
Anaerobic sludge	rice	5.9 ^b	51%	28%	1%	20%	/	/	/	/	/	/	(Dong et al., 2009)
	potato	4.7 ^b	51%	38%	7%	3%	/	/	/	/	/	/	
	lettuce	2.2 ^b	67%	26%	2%	5%	/	/	/	/	/	/	
Mixed anaerobic sludge	sorghum stalks	2 ^b	0%	44%	30%	26%	/	/	/	/	/	/	(Shi et al., 2009)
	sorghum stalks	5 ^b	0%	25%	53%	22%	/	/	/	/	/	/	
Enriched culture from cracked cereal	grass	0.2 ^b	64%	7%	4%	25%	/	/	/	/	/	/	(Cui and Shen, 2012)
Enriched culture from	soybean	0.2 ^c	11%	34%	3%	19%	/	/	33%	/	/	/	(Han et al., 2012)

Inoculum	Substrate	H ₂	HAc	HBut	EtOH	HPr	HLac	HFor	HVal	HCap	HSuc	Major Bacteria	References
cracked cereal	straw												
	soybean	0.5 ^c	15%	31%	2%	17%	/	/	35%	/	/	/	
	straw												
	soybean	1 ^c	13%	38%	1%	16%	/	/	32%	/	/	/	
	straw												
Anaerobic activated sludge	rice straw	6 ^d	67%	25%	7%	/	/	/	/	/	/	<i>Clostridium sp.</i>	(Cheng et al., 2011)
<i>C. butyricum</i> AS1.209	Corn straw	2.6 ^c	46%	28%	7%	19%	/	/	/	/	/	<i>C. butyricum</i> AS1.209 (%)	(Li and Chen, 2007)
Mesophilic digested sludge	Starch	6.1 ^e	15%	80%	2%	0%	0%	3%	/	/	0%	<i>C. acetobutylicum</i> ; <i>C. butyricum</i>	(Akutsu et al., 2009a)
Soybean meal	Starch	5.6 ^e	27%	40%	31%	0%	0%	2%	/	/	0%	<i>C. acetobutylicum</i> ; <i>C. butyricum</i>	
Kitchen Waste	Starch	5.1 ^e	20%	71%	2%	0%	0%	6%	/	/	1%	<i>C. paraputrificum</i> ; <i>Citrobacter freufii</i>	
Cattle Manure	Starch	4.6 ^e	23%	49%	22%	0%	0%	7%	/	/	0%	<i>C. acetobutylicum</i> ; <i>C. butyricum</i>	
Activated Sludge	Starch	3.5 ^e	36%	5%	54%	2%	0%	3%	/	/	0%	<i>Bacteroides eggerthii</i> ; <i>C. pasteurianum</i>	
Thermophilic Digested Sludge	Starch	2.2 ^e	42%	7%	51%	0%	0%	0%	/	/	0%	<i>Clostridium sp.</i> ; <i>Bacillus sp.</i>	
Soil	Starch	2 ^e	31%	2%	51%	10%	0%	2%	/	/	4%	<i>Bacteroides eggerthii</i>	
Thermophilic Acidogenic Sludge	Starch	0.7 ^e	22%	0%	66%	0%	1%	4%	/	/	7%	<i>Bacteroides sp.</i> ; <i>C. aminovalericum</i>	
Anaerobic sludge	Wheat bran	/	16%	32%	/	19%	/	/	33%	/	/	/	(van Aarle et al., 2015)
Granular sludge	Wheat bran	/	0%	37%	/	22%	/	/	40%	/	/	/	
Acidogenic fermentation broth	Wheat bran	/	13%	30%	/	24%	/	/	33%	/	/	/	

Such as metabolites composition analysis, analyses of bacterial community structure are not always performed which makes difficult to determine whether differences of hydrogen amounts are due to metabolic shifts and/or different bacterial community structures. This knowledge gap about structure composition of mixed culture makes difficult to link inoculum bacterial composition to fermentative metabolism and so to hydrogen potential. In conclusion, mixed cultures functioning remain a black-box for which structure-function relationship is still misunderstood. Nonetheless, recent advances in molecular biology offer new possibilities to resolve this paradigm (Koch et al., 2014). Such methods are presented in the next section.

1.5.2 Analytical technics to understand structure-function relationship in mixed culture

Over recent years, novel technics to identify microorganisms thanks to their DNA composition have been developed. High-throughput sequencing analysis have been democratised, offering the possibility to rapidly sequence high number of microbial DNA (Caporaso et al., 2012). But such analysis is very expensive and sequencing the full genome of all microorganisms present in inoculum may reach extreme prices.

Bacterial species can be differentiated by targeting some specific sequences, very different from one species to another, called hypervariable region. These sequence portions are especially present on the 16S rRNA subunit sequence. Main advantages of 16S rRNA genes to differentiate prokaryote are that these sequences are distributed in all bacteria and archaea, and present highly conserved and hypervariable regions (Mizrahi-Man et al., 2013). Nine hypervariable regions, namely V1 to V9 regions, are present in prokaryotes and can be used to differentiate the microbial species. Conserve regions are used to target common bacterial sequences encircling hypervariable regions. The amplified fragments can then be differentiated using denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) or new generation sequencing methods as presented before to affiliate bacteria to the closest taxonomic rank.

The number of different hypervariable regions used to identify bacterial specie changes according studies. Recent reports showed that intragenomic heterogeneity widely spread in 16S rRNA subunit, can lead to an overestimation of the diversity (Sun et al., 2013). Analysis of the partial region is thus preferred since it generates less overestimation. In that case, only 23% of heterogeneity is estimated at the unique level of V3 region against 123.7% for the full length 16S RNA subunit. However, the use of only one region does not allow covering the full phylogenetic affiliation to genus rank. Mizrahi-Man *et al* (2013) showed that the percentage of coverage decreases

with the phylogenetic rank. According to their study, only regions V3 and V4 lead to efficient phylogenetic affiliation, with 96, 95, 92, 68 and 54% of coverage for phylum, class, order, family and genus affiliation respectively for the V3 region.

Analysis of V3 16S rRNA region can provide a visualisation of the bacterial community structure. With this tool, some authors followed the community structure dynamics to interpret metabolic changes. By sequencing the V3 16S rRNA region at different times of continuous production of hydrogen, Huang *et al* (2010) observed different bacterial community structures during the study of continuous production of hydrogen using cattle dung compost. The starting phase was characterised by the presence of *Bacillus* sp. which were replaced by *Clostridium perfringens* and *Clostridium beijerinckii* when hydrogen production was maximal. The community shifted to *C. lundens*, *C. peptidivorans* and *C. vincentii* during the decline phase. Because no analysis of the metabolites was performed, it is not possible to know whether the community structures shift led to a metabolic shift.

In addition, a complete analysis of the dark fermentation process was realised by Romano *et al.* (2014) by using heat treated and non-heat treated lake sediment on glucose. Authors observed that without heat treatment, many type of bacteria grown during the fermentation, i.e. five species affiliated to *Clostridium* sp. representing 95% of all the bacteria, bacteria affiliated to *Bacillus* sp., *Lactobacillus* sp. and *Sporolactobacillus* sp. (5% of abundance). Even though acetate and butyrate mainly accumulated, lactate was also produced. The heat treatment of the inoculum resulted in selection of mainly one bacteria *C. bifermentans*, 92/96% of abundance. This community structure change conducted to a significant decrease of lactate production to the benefit of ethanol.

1.6 Conclusion and aim of the thesis

This literature review shows that hydrogen is a clean energy vector that can be potentially produced by the conversion of lignocellulosic residues. During the process of dark fermentation, fermentative microorganisms hydrolyse the complex organic matter and convert carbohydrates into hydrogen and metabolites. The potential for hydrogen production is closely related to the nature and composition of the biomass. Generally, high production of hydrogen was obtained from soluble sugars with butyrate producing pathway as main metabolism. Accumulation of formate, lactate or ethanol was observed in the case of low hydrogen production. Overall, the use of more complex substrate resulted in poorest substrate conversion and low accumulation of hydrogen. The production of propionate, associated to no hydrogen production, was observed during the fermentation of the majority of complex substrates. One solution could be to associate a

pretreatment that can improve the production of hydrogen by simplifying the matrix with more or less efficiency depending on the pretreatment methods.

However, the composition of the bacterial community is also a key factor to the production of hydrogen. Less amounts of gas are obtained if hydrogen-consuming microorganisms are selected during the bio-process. Bacteria affiliated to *Clostridium sp.* are generally targeted since they show efficient production of hydrogen, contrary to *Bacillus sp.* mostly known to reuse H₂. But the selection of bacteria during fermentation mainly depends on the substrate composition and cannot be dissociate. Indeed, microorganisms did not have the same ability to degrade biomass. It can result on different final structure of bacterial community at the end of the process or in the development of different metabolic patterns. A lack of information on the link between microbial community and metabolic profiles according to the substrate is missing in the literature. It is difficult to link the structure of bacterial community developed in reactors with the composition of the substrate fermented and the global metabolism of the culture. Such information could lead to better understanding on how mixed cultures work during dark fermentation to then propose efficient solution to conduct the process.

Chapter 2. Materials and methods

2.1 Biological process for hydrogen production

2.1.1 Source of mixed microbial communities

Six different microbial communities were used as initial microbial inoculum in this thesis.

The outlet of a solid-state anaerobic reactor fed with bull manure (Montrodat, France), so-called digestate, was used. Because undigested straw was present in the digestate, microorganisms were first extracted according to a protocole adapted from Zhang *et al* (2007). For that, 40 ml of sterile physiological water were added to 20 g of digestate in a 500 ml centrifuge tube. After dynamic hand shaking, the tube was centrifuged for 5 min at 3000 g (temperature 4°C). The supernatant was recovered in a sterile bottle and the pellet was washed with 40 ml of sterile phosphate buffer at 10 mM. After shaking, the tube was again centrifuged at 3000 g for 10 min (4°C). The supernatant was recovered and added to the first one. The residual juice of the pellet was filtered at 1 mm and was added to the supernatant. This liquid mixture containing microorganisms was used as inoculum. One part was directly used after extraction and the inoculum was called "**Fresh Digested Manure**" (**FM**). The other part was named "**Stored Manure**" (**StM**) since it was used as inoculum after a storing period of 2 months at 35°C without feeding.

Aerobic sludge was sampled in January 2015 at the Narbonne Waste Water Treatment Plant (WWTP) in the biological treatment tank. One portion of the sludge was used for dark fermentation directly after sampling at the WWTP and was called "**Fresh Aerobic Sludge**" (**FAS**), whereas this other part was stored without feeding for 2 months at 35°C and was named "**Stored Aerobic Sludge**" (**SAS**). Finally, a fifth inoculum was sampled in the same WWTP but in the storage tank of return activated sludge, here called "**Fresh Settled Aerobic Sludge**" (**FSAS**).

A sixth inoculum was represented by fresh aerobic sludge but sampled in April 2015 in the same WWTP (**FAS-Ap**). It was used as fermentative bacterial consortium for fed-batch experiment realised during the fifth chapter of the thesis.

All inocula were heat-shock pretreated before inoculation in reactors in order to inhibit methanogenic archaea activity. For that, inocula were first warm up to 90°C for 30 min and instantly ice cooled. They were inoculated in reactor once at room temperature.

2.1.2 Biohydrogen production in batch tests

Biological hydrogen potential tests (BHP-tests) were performed in batch reactors for the third and fourth chapters of the thesis.

In the third chapter, i.e. “Impact of the substrate type on fermentation”, the fermentation of seven substrates was performed with Fresh Manure (FM) and Stored Manure (StM) inocula. Carbohydrates were selected as models of the lignocellulose fractions. Cellulose was represented by microcrystalline cellulose (MCC) (Avicel, Fluka), cellobiose as repeating unit in cellulose (Fluka), and glucose as cellobiose monomer (Sigma). Hemicelluloses were represented by xylan as pentose polymer (Sigma), and arabinose and xylose as monomers (Sigma). Finally, wheat straw was chosen as model of lignocellulosic residue (Hausmann common wheat straw, grounded fraction between 0.4 and 1mm).

For the fourth chapter, “Relationships between initial and final bacterial community structures, and metabolisms identified after dark fermentation”, glucose was selected as the easiest biodegradable carbohydrate to study the effect of inoculum bacteria community structure on dark fermentation mechanisms.

All batch-tests were carried out by quadruplicate in 600 ml plasma bottle previously sterilised. 400 ml of working volume were composed of 12.5 ml of minimal medium (in $\text{mg}\cdot\text{l}^{-1}$ - NH_4Cl : 32000 ; K_2HPO_4 : 20000 ; $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$: 1500 ; $\text{H}_3\text{BO}_3\cdot \text{H}_2\text{O}$: 60 ; $\text{MnSO}_4\cdot \text{H}_2\text{O}$: 117 ; $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$: 25 ; ZnCl_2 : 70 ; $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$: 25 ; $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$: 15 ; $\text{NaMoO}_4\cdot 2\text{H}_2\text{O}$: 25 ; HCl : 1755). MES (2-[N-morpholino] ethane sulfonic acid) buffer at 100 mM was used to maintain pH value at 6. The minimal medium and buffer solution were filtered through 0.2 μm before being introduced into the flask.

The substrate concentration was set according to the residue complexity and its difficulty to be degraded and converted into by-products. Hence, a concentration of 5 $\text{g}_{\text{COD}}\cdot\text{l}^{-1}$ was used for glucose, cellobiose, arabinose, xylose and xylan. A concentration of 10 $\text{g}_{\text{VS}}\cdot\text{l}^{-1}$ was fixed for MCC and wheat straw in order to accumulate enough gas in the medium to exceed the limit of detection of the analytical device.

Heat treated inoculum was added at last, in quantity to obtain a substrate on inoculum (S/X) ratio of 10 $\text{g}_{\text{substrate eq. COD}}\cdot\text{g}_{\text{inoculum eq. COD}}^{-1}$ for all experiments. After inoculation, pH was adjusted at 6.0 with 32% NaOH (Sigma), bottles were sealed with a rubber stopper and locked with an aluminium

screw. Head space was then purged with 0.2 μ m-filtered nitrogen gas to remove oxygen traces and set anaerobic condition.

Gas production was followed by regular gas analysis by micro-gas chromatography (μ -GC), i.e. every eight hours. Fermentation was stopped when the production of hydrogen reached the plateau of production for one replicate in order to avoid further consumption of the gas. At this time, reactors were opened and 20 ml of medium were kept for metabolites and microbial community structure analysis.

2.1.3 Dark fermentation in fed-batch reactor

The process of fed-batch was used in the fifth chapter, to favour the emergence of both hydrogen producing and hydrolytic bacteria. The selection pressure on substrate was applied with glucose homo-polymers as sole carbon sources, in order to simplify the system. Semi-amorphous cellulose, named SAC (BIOFLOC96, Tembec), was used as polymer of cellobiose to promote activity of hydrolytic bacteria. It was preferred to micro-crystalline cellulose as it presents 50% of amorphous regions that are easier accessible to hydrolytic enzymes than crystalline ones. In addition, cellobiose (glucose dimer) was used as easy degradable carbohydrate to promote the selection of hydrogen producing bacteria. It was preferred to glucose to maintain hydrolysis activity since a hydrolysis step, simpler than required for SAC, is necessary for its conversion.

Fed-batch dark fermentation was performed with only cellobiose (CB reactors), only SAC (SAC reactors), and with a mix of SAC and cellobiose (MIX reactors) to observe the synergetic effect of co-fermentation of these two substrates.

As for batch-tests, experiments were performed in quadruplicates, in sterile 600 ml plasma bottles with 200 ml of initial working volume. The medium was composed of 5.5 ml of minimal medium (in mg.l⁻¹ - NH₄Cl : 32000 ; K₂HPO₄ : 20000 ; FeCl₂.4H₂O : 1500 ; H₃BO₃.H₂O : 60 ; MnSO₄.H₂O : 117 ; CoCl₂.6H₂O : 25 ; ZnCl₂ : 70 ; NiCl₂.6H₂O : 25 ; CuCl₂.2H₂O : 15 ; NaMoO₄.2H₂O : 25 ; HCl : 1755), and 100 mM of MES buffer.

At start of the experiment, 10 g_{VS}.l⁻¹ of SAC were introduced in **SAC** reactors, 8 g_{VS}.l⁻¹ of SAC and 2 g_{substrate eq. COD}.l⁻¹ of cellobiose in **MIX** reactors, and 2 g_{substrate eq. COD}.l⁻¹ of cellobiose in **CB** reactors. Then, the inoculum (FAS-Ap) was added in the medium after heat-shock pretreatment to have a S/X ratio of 10, and the pH was adjusted to 6.0 with 32% NaOH (Sigma). Bottles were sealed with a

rubber stopper and locked with an aluminium screw. Finally, head space was purged with 0.2 μ m-filtered nitrogen gas to install anaerobic conditions.

Gas production was measured every eight hours by μ -GC. Once the cumulative hydrogen reached a plateau of production, 10 ml of medium were sampled in each reactor for metabolites and microbial community structure analysis. Then, 50 ml of new medium with the same composition as before was added. Only 2 g_{substrate eq. COD}.l⁻¹ of cellobiose was supplemented in cellobiose-based reactors, i.e. CB and MIX reactors, to stimulate the activity of hydrogen producing bacteria. At the contrary, no more SAC was added in media since this substrate was not totally consumed during the time of experiment.

This feeding procedure was carried out until reactors received six supplies, corresponding to six feeding cycles (C1 to C6). At the end of the sixth cycle, 20 ml of medium were kept for metabolites and microbial community structure analysis.

2.1.4 Summary of the experimental conditions with references to chapter numbering

The different experiments performed during the thesis are summarised on Table 2.1-1.

Table 2.1-1: Summary of the different conditions used for the experiments performed in the thesis chapters.

Chapters	Type of reactor	Type of inoculum	Type of substrate	Initial substrate concentration	Feeding substrate concentration	S/X ratio
Chapter 3) Impact of the substrate type on fermentation	Batch	FM StM	Glucose; Cellobiose; Arabinose; Xylose; Xylan	5 g _{eq. COD} .l ⁻¹	/	10
			MCC; Wheat straw	10 g _{vs} .l ⁻¹		
Chapter 4) Relationships between initial and final bacterial community structures, and metabolisms identified after dark fermentation	Batch	FM; StM; FAS; SAS; FSAS	Glucose	5 g _{eq. COD} .l ⁻¹	/	10
Chapter 5) Selection of hydrolytic cultures	Fed-batch	FAS-Ap	SAC	10 g _{vs. COD} .l ⁻¹	/	10*
			Cellobiose	5 g _{eq. COD} .l ⁻¹	2 g _{eq. COD} .l ⁻¹	

able to perform hydrogen production by dark fermentation			SAC + Cellobiose	8 g _{VS} .l ⁻¹ + 2 g _{eq. COD} .l ⁻¹	/ +	2 g _{eq. COD} .l ⁻¹	
---	--	--	------------------------	--	--------	---	--

*Value for the first fed-batch cycle

2.2 Biochemical and biological analysis

2.2.1 Biochemical analyses

2.2.1.1 Total Solids and Volatile Solids, and Chemical Oxygen Demand analysis

Total Solids (TS), Volatile Solid (VS) and Chemical Oxygen Demand (COD) analysis were periodically quantified on substrates and on inocula in order to realise the experiments.

VS analysis was done on inocula, wheat straw, MCC and SAC in quadruplicates according to the APHA standard methods (APHA, 1999). Clean porcelain crucibles were first put in 550°C oven over night in order to remove any residual volatile particles. Once at room temperature, about 10 g of matter was exactly weight in crucible. After 105°C evaporation for 24 h wheat straw, MCC and SAC, and 48 h for inocula, crucibles were again weighted. The residual mass corresponded to the Total Solid (TS) content of the matter. Then crucibles were put at 550°C for 3h to burn the organic matter. After cooling at room temperature, the new crucible mass measured was subtracted from the mass before 550°C combustion. This value corresponded to the Volatile Solid (VS) content of the matter.

Chemical oxygen demand (COD) was evaluated for soluble sugar solutions, i.e. glucose, cellobiose, arabinose, xylose and xylan. It was analysed by spectrophotometric method using Spectroquant® kit (Merk) according to the manufacturer's indication. A volume of 2 ml of prepared solution was introduced in COD-tube that was heated for 2h at 150°C. During the reaction, the organic matter is oxidised by a solution of potassium dichromate with silver sulphate as catalyser. The oxidation process releases Cr³⁺ ions which are dosed by spectrophotometry at wavelength of 620nm once the solution is at room temperature.

2.2.1.2 Analyses of gas and metabolites produced

Gas production was monitored every 8 hours with an automatic micro-gas chromatograph (SRA μ-GC R3000) equipped with two columns: a Molsieve 10m/PPU at 80°C with Argon as vector gas and a VAR 8m/PPU at 70°C with Helium, for O₂-CH₄-H₂-N₂ and CO₂ analysis, respectively. The TCD temperature was set at 90°C. Gas production was estimated by pressure measurement.

Soluble metabolites, i.e., volatile fatty acids (VFAs) and organic acids, solvents and residual carbohydrates, were quantified before and after fermentation. VFAs, i.e. acetate, butyrate, isobutyrate, propionate, valerate, isovalerate and caproate, were measured by Gas Chromatography (Perkin Clarus 580) equipped with an injector heated at 250°C, a capillary column (Crossbond® Carbowax®, 15m) heated at 200°C and flame ionization detector (FID) heated at 280°C). Carrier gas was nitrogen injected at 6 mL.min⁻¹. Two mL of liquid were sample from reactors and centrifuged at 5000g during 10 min (Eppendorf®, Mini spin), Then, 600 µl of the supernatant were put in analytical vials with 600 µl of standard internal solution (1 g.L⁻¹ of 2-Ethylbutyric acid - C₂H₅CH(C₂H₅)COOH).

Residual carbohydrate, i.e. glucose, arabinose and xylose, and organic acids, i.e. succinate, formate, lactate and ethanol, were measured by High Performance Liquid Chromatography (HPLC). The HPLC was coupled to refractometric detection (Waters R410). Chemicals were separated by an Aminex HPX-87H column (300 x 7.8mm, Biorad) equipped with a protective precolumn (Microguard cation H refill catbridges, Biorad). The eluting solution corresponded to 2 mM H₂SO₄ under a flow rate of 0.4 mL.min⁻¹. The column temperature was set at 35°C and the refractive index detector (Waters 2414) worked at 45°C. In this case, samples were filtrated at 0.2 µm prior to analysis.

2.2.2 Biological analyses

2.2.2.1 Sample collection

Ten mL of liquid sample were collected before and after the fermentation test. Samples were first centrifuged for 10 min at 5,752 g. After removing 8 ml of the supernatant, the pellet was resuspended and aliquots of 500 µl were introduced in 4 sterile eppendorf tubes of 2 ml. The tubes were then centrifuged 5min at 16,662 g. The pellets were finally stored at -20°C prior to DNA extraction.

2.2.2.2 DNA extraction and purification

DNA extraction protocole was adapted from Godon et al. (1997). Frozen pellet were resuspended in 385µl of 4M guanidine thiocyanate–0.1 M Tris (pH 7.5), 115µl of 10% N-lauroyl sarcosine and 500µl of N-lauroyl sarcosine–0.1 M phosphate buffer (pH 8.0). After vortex homogenisation, the 2 ml tube was incubated at 70°C for 1h. At the end, tubes were put in ice and one volume (750 µl) of 0.1-mm-diameter silica beads (Sigma) was added. The tubes were shaken at maximum speed for 30s in a Vibro shaker (Retsch). Polyvinylpyrrolidone (15 mg) was added to

the tube, which was vortexed and centrifuged for 3 min at 12000g. After recovery of the supernatant, the pellet was washed with 500µl of TENP (50mM Tris [pH 8], 20mM EDTA [pH 8], 100mM NaCl, 1% polyvinylpolypyrrolidone) and centrifuged for 3 min at 12000g. The new supernatant was added to the first one and the washing step was repeated three times. The pooled supernatants (about 2 ml) were briefly centrifuged to remove particles and then split into two 2-ml tubes. Nucleic acids were precipitated by the addition of 1 volume of isopropanol for 10 min at room temperature and centrifuged for 15 min at 20000g. Pellets were resuspended and pooled in 200µl of sterile water. 20µl of ARNase (1mg/ml) were added and the tubes were incubated at 37°C for 10 min.

DNA purification was realised with kit QIAamp DNA Mini Kit (QIAGEN). AL buffer (200µl) was added to the tube which was incubation 10min at 70°C. Then, 200µl of ethanol for molecular biology was added and the content was transferred on the QIAamp column. After centrifugation (1min, 20000g), 500µl of AW1 buffer was added to the column which was then centrifuged in the same condition. Then 500µl of AW2 buffer was added and the column was centrifuged 3min at 20000g and 1 min more to avoid all contamination with the buffer. DNA on the column was solubilised by adding 50µl of pure sterile water and was recovered by 1min of centrifugation (8000RPM). DNA fragment were quantificate with the Infinite M200 Reader (TECAN).

2.2.2.3 Polymerase chain reaction (PCR) amplification and Illumina Sequencing (High-throughput amplicon sequencing)

The V3 and partial V4 region of the 16S rRNA gene were amplified using the primer CTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG and the reverse primer GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT plus the respective linkers over 30 amplification cycles at an annealing temperature of 65.0 °C. In a second PCR reactor of 12 cycles, an index sequence was added using the primers AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGAC and CAAGCAGAAGACGGCATAACGAGAT-index-GTGACTGGAGTTCAGACGTGT.

The PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions for high throughput amplicon sequencing of paired 458 bp reads (V3-V4 chemistry). Library preparation and sequencing was done at the GeT PlaGe Sequencing Center of the Genotoul Lifescience Network in Toulouse, France.

A modified version of the Standard Operation Procedure for MiSeq data (Kozich *et al.*, 2013) in Mothur version 1.35.0 (Schloss *et al.*, 2009) was used to assemble forward and reverse sequences.

Sequences were pre-clustered at 4 differences in nucleotides over the length of the amplicon and chimeras were checked using uchime (Edgar *et al.*, 2011). Alignment and taxonomic affiliation from the 16S rRNA sequences was performed on the totality of samples sequenced with SILVA SSU Ref NR99, release 119, as provided by Mothur (Schloss *et al.*, 2009).

2.3 Data analyses

2.3.1 Determination of kinetic parameters of the H₂ production

From the gas composition and volume analysis, a modified Gompertz model was used to assess hydrogen production kinetic parameters (Equation 7):

$$\text{Equation 7: } H(t) = P \exp \left\{ -\exp \left[\frac{Rm \cdot e}{P} (\lambda - t) + 1 \right] \right\}$$

Where H is the cumulative volume of hydrogen production (ml) along the incubation time (t), P is the maximum cumulative hydrogen production (ml_{H₂}·g⁻¹_{eq initial COD}), Rm is the maximum hydrogen production rate (ml_{H₂}·g⁻¹_{eq initial COD}·d⁻¹), λ is the lag phase (days) and e is exp(1). The values of P, Rm and λ were estimated using *grofit* R package (v 1.1.1-1) with nonlinear least square fitting (Kahm *et al.*, 2010).

2.3.2 Multivariate analysis performed on metabolic patterns and bacterial community structure – Principal Component Analysis (PCA)

Principal component analysis (PCA) was used as multivariate analysis to explore metabolic patterns and bacterial community structures observed during dark fermentation in Chapters 3 and 4. The mathematical algorithm used in PCA creates an orthogonal vectorial base from a set of observations of possibly correlated variables (Ringnér, 2008). The new uncorrelated variables are called principal components. The first principal component is defined to explain the largest possible variance. Then, each succeeding component are defined to explain the highest possible remaining variance under the constraint that it is orthogonal to the preceding components. The PCA is so used to reduce the number of variables and reduce redundancy, which simplifies data interpretation.

PCA was performed using the “*prcomp()*” function from the built-in R stats package. Graphic representation was performed using “*ggbiplot*” (Vu, 2011).

In the case of metabolic patterns analysis, PCA was carried out on reduced and scaled data expressed as $\text{g}_{\text{metabolite eq. COD}} \cdot \text{g}_{\text{initial COD}}^{-1}$ in Chapter 3 in order to introduce the biodegradation potential aspect in the analysis. In Chapter 4, PCA was carried out on reduced and scaled data expressed as $\text{g}_{\text{metabolite eq. COD}} \cdot \text{g}_{\text{converted COD}}^{-1}$ to observed the metabolic pattern effect.

For bacterial community structure analysis, Operational Taxonomic Units (OTUs) at unique level obtained after treatment of sequences were used to perform PCA. However, rare species present in microbial communities largely influence such analysis. Indeed, data transformation was proposed to reduce the influence of rare species without affecting results. Hellinger transformation was proposed as efficient transformation before calculation of Euclidean distance matrix used for PCA (Legendre and Gallagher, 2001). Because of this, sequence data set was first transformed by Hellinger method using the “*decostand()*” function from the *vegan* R package (Oksanen *et al.*, 2016) prior to PCA.

In Chapter 3, PCA was also utilised to confront the structure of bacterial community observed in each reactor with the metabolic patterns developed during dark fermentation. Influence of major OTUs identified on metabolites produced was analysed by the “*envfit()*” function (permutations 9999) which fits environmental vectors onto ordination, PCA done on major identified OTUs (>5%) in this case (Oksanen *et al.*, 2016).

2.3.3 Identification of bacterial community structure gradient with Nonmetric multidimensional scaling (NMDS)

In Chapter 5, Nonmetric Multidimensional Scaling (NMDS) was performed to describe the bacterial community changes in fed-batch experiments. Like PCA, NDMS aims to group information from multiple dimensions into generally 2 dimensions to simplify data visualisation and interpretation. But contrary to PCA, distance between samples projected on a plot is calculated according to samples rank order and not to their metric distance. Hence, the distance between two neighbouring points on NMDS plot represents the similarity between these objects, but not the original distance (Ramette, 2007). Because of this, NMDS analysis has been proposed as efficient technique to identify gradients (Paliy and Shankar, 2016). In the case of fed-batch experiments, change of bacterial community according a time gradient was targeted. NMDS was realised using Bray-Curtis distance with the “*ordinate()*” function from the *phyloseq* R package (McMurdie and Holmes, 2014).

2.3.4 Searching for correlations between metabolic patterns and bacterial families present in inocula and selected mixed cultures

In the fourth chapter, relationship between metabolic patterns identified at the end of dark fermentation and bacterial community structure of inoculum and selected mixed culture were attempted to be found.

For this, Mantel test was first used to identify correlations between these three data sets. The test compares the distance matrixes in pairs under the null hypothesis H0 that 'the two matrixes X and Y are not correlated'.

$$\text{Equation 8: } r_M = \frac{1}{d-1} \sum_{i=1}^{n-1} \sum_{j=i+1}^n \left(\frac{x_{ij}-\bar{x}}{s_x} \right) \left(\frac{y_{ij}-\bar{y}}{s_y} \right)$$

With r_M the Mantel's statistic, i and j numbers of rows and columns of matrixes, $d=[n(n-1)/2]$ number of distance calculated in the upper triangular portion of each matrix, and s the standard deviation for variables x and y .

The r_M value is then confronted to a probability of reference (p_{ref}) under H0. Indeed, if r_M is higher than p_{ref} , H0 is rejected and the alternative hypothesis H1 "the two matrixes are correlated" is considered as the hypothesis that best explains data sets. Significance of the test was evaluated by the realisation of 9999 permutations. Mantel's test was realised using "*mantel()*" function from *vegan* R package.

Then, correlations were attempted to be found between these three data sets using metabolic patterns and biological indicators calculated from bacteria family affiliation in inocula and selected mixed culture communities. For more clarity, the choice and calculation of these indicators will be explain in Chapter 4. Correlation were evaluating by regularised Canonical Correlation Analysis (rCCA) that calculates linear combinations between two independent matrixes. The rCCA was realised using "*rcc()*" function from the *mixOmics* R package (Le Cao et al., 2015).

2.3.5 Summary of statistical tests used in this thesis

All statistical tests described above were selected to demonstrate a specific result. Table 2.3-1 was realised to sum up and better visualise the interest of each analysis.

Table 2.3-1: Summary of statistical test realised in for the thesis.

Type of test	Aim of test	Aim for the thesis	Chapter
Principal Component Analysis (PCA)	Multivariate analysis that summarises the variance in low-dimensional scale. Allows to identify groups, trends or key variables. Distance between object represents the dis-similarities observed between them	1- To group reactors according metabolic patterns or selected bacterial community structure identified	Chapter 3 and 4
		2- To fit metabolic patterns with selected bacterial community structure	Chapter 3
Mantel's test	Statistical test to evaluate correlation between two independent matrixes	To show the presence of correlation(s) between metabolites produced and initial and final bacterial community structures	Chapter 4
Regularised Canonical Correlation Analysis (rCCA)	Multivariate analysis that calculates linear correlations between two data sets. This method is more interpretive than PCA which is more explorative.	To evaluate linear combinations between metabolic patterns and biological indicators designed to explore the initial and final bacterial community structures	Chapter 4 and 5
Nonmetric multidimensional Scaling (NMDS)	Gradient analysis approach based on distance matrix calculated from object rank order. The main drawback is that information about original variable weight is lost, but this method is more robust when data do not have an identifiable distribution	To explore bacterial community structure dynamic along fed-batch cycles and to identify temporal gradient	Chapter 5

***Chapter 3. Impact of the substrate type on
fermentation***

4.1 Context and objectives

Lignocellulose is a complex organic matter mostly composed of phenol- and carbohydrate-based polymers. Lignin is the polyphenolic component of the biomass which degradation under anaerobic conditions is generally observed after long incubation period of at least more than seven months (Benner et al., 1984). Hemicelluloses are polymers which mainly consist in pentose-based sugars, e.g. arabinose and xylose, whereas cellulose is composed of only hexose-based sugars, i.e. glucose units (Menon and Rao, 2012). In the context of dark fermentation, only these two last lignocellulosic polymers have been reported to be converted into hydrogen.

Previous studies on lignocellulosic residues have shown that the polymerisation degree of carbohydrates are structural features of the biomass that are barriers to the production of hydrogen (Guo et al., 2014). Since the hydrolysis step requires high energy and the reduction of biochemical intermediates, i.e. NADH to release carbohydrates from polymers, only the fermentation of free sugars can lead to the production of high amount of hydrogen. Looking at the amount of hydrogen accumulated from various sugars, it was reported in the literature that H₂ production mainly depended on the type of carbohydrate, e.g. pentose or hexose-based sugars (Mäkinen et al., 2012; Quéméneur et al., 2012b). Such difference can be explained by both the metabolic patterns occurring during the bioprocess and the structure of the bacterial community when using mixed cultures (Akutsu et al., 2009a).

Up to now, only few works have tried to link the production of hydrogen with related metabolic patterns to the type of microorganisms present in the consortium, regarding the substrate. The tri-interaction between substrate composition, metabolic patterns and organisation of bacterial communities has already been suggested in several works but only few studies performed a deep analysis to resolve this paradigm. Akutsu *et al.* (2009a) worked on complex substrates, i.e. starch, protein and lipids, and used eight different types of mixed microbial cultures. Meanwhile, Quéméneur *et al.* (2012b) used only one type of inoculum and compared the fermentation of hexose-based carbohydrates with different polymerisation degrees. In both studies, the composition of the substrate drove the final community structure which was associated to different metabolic patterns and so to different hydrogen production yields.

This chapter aims to investigate the influence of carbohydrate molecular composition on dark fermentation microbial mechanisms. The fermentation of seven substrates representing the main

molecules and polymers composing the lignocellulose was studied using two mixed bacterial cultures sampled from manure anaerobic digestate.

A first set of experiments was performed in batch tests using bacteria freshly extracted from manure anaerobic digestate, inoculum called *Fresh Manure (FM)*, and after heat shock treatment to inhibit the methanogenic activity. Micro-crystalline cellulose (MCC), cellobiose and glucose were used as substrates, to represent the cellulosic fraction with different degrees of polymerisation. Hemicelluloses were represented by pentose-based sugars, ie. arabinose, xylose and the polymeric form, xylan. Wheat straw was the seventh substrate used as the most complex model of lignocellulosic biomass.

A second set of experiments was carried out in the same conditions, using the same substrates but with an inoculum having two months of storage at 35°C with no feeding, so called *Stored Manure (StM)*. Heat shock treatment was also performed on the mixed culture after storage, just before reactors inoculation. Room temperature storage is often preferred before fermentation to exhaust the carbon sources. Indeed during transportation or experiment implementation, residues of organic matter already present in the medium are slowly consumed. This step can change the initial microbial composition and therefore the final fermentation behaviour. The impact of such storage period was evaluated during the second experiment.

For the two experiments, batch tests were similarly performed as explained in 2.1.2. The production of hydrogen was monitored until a plateau was reached to minimise H₂ consumption. Metabolites that accumulated meanwhile in the liquid phase were analysed at the end of the process to determine the metabolic pathway involved according to substrate conversion. Bacterial DNA was extracted from each end-culture and was sequenced through high-throughput sequencing to assess the structure of the final bacterial communities.

In this chapter, dynamics of hydrogen production is first presented for the fresh mixed culture (FM) and according to each substrate. Related metabolic patterns are also discussed considering the relationship between the hydrogen production yield and the bacterial community structure developed at the end of the process that was confronted to fermentative metabolisms.

Same analysis was performed on the stored inoculum (StM) to observe the influence of inoculum storage on substrate conversion, i.e. into hydrogen and other metabolites, and on final bacterial community structure.

4.2 Impact of substrate composition on dark fermentation performed inoculated with a fresh digested manure (FM)

Adapted from "Chatellard et al., 2016, The type of carbohydrates specifically selects microbial community structures and fermentative patterns, Bioresource Technology, Accepted for publication on September 17th 2016, DOI: 10.1016/j.biortech.2016.09.

4.2.1 Hydrogen production performances

By using a fresh digested manure (FM) inoculum, the maximum of hydrogen accumulation was reached at different fermentation time for each substrate in accordance to their biodegradability. Indeed, glucose, cellobiose, arabinose and xylose fermentation were stopped after 4.5, 6.4, 6.5, and 7.5 days respectively, while longer time was required for MCC, xylan and wheat straw, which were stopped after 21.5, 8 and 14.6 days, respectively. No methane production was observed during the experiment showing that the heat shock pre-treatment totally suppressed the methanogenic activity for the time of experiment (Figure 4.2-1).

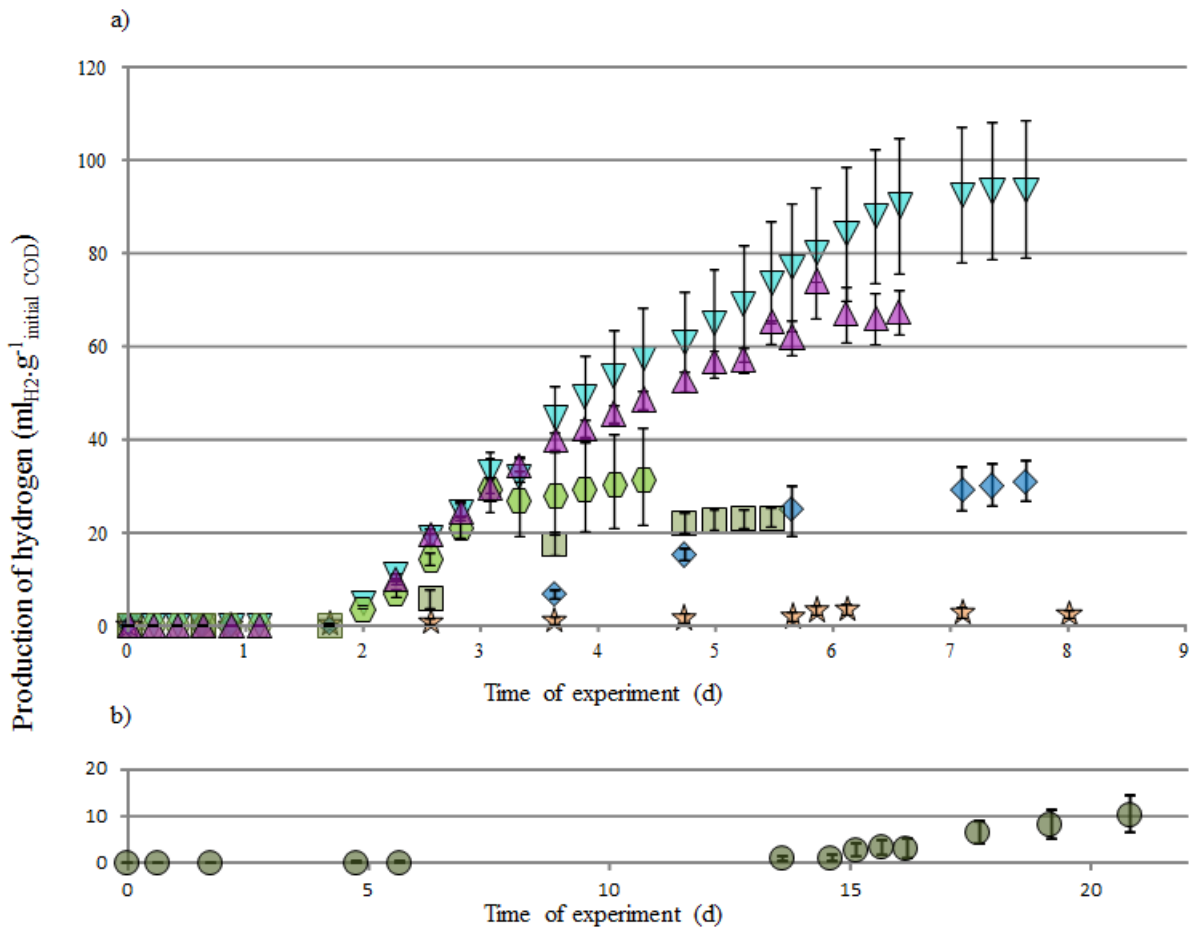


Figure 4.2-1: Production of hydrogen analysed for each substrate during the experiment ml_{H2}·g⁻¹ of initial substrate eq.COD for a) glucose (●), cellobiose (■), arabinose (▲), xylose (▼), xylan (◆), and wheat straw (★), and b) for MCC (●).

Dynamics of hydrogen production along the experiment are presented in Figure 4.2-1. A modified Gompertz model was fitted to hydrogen production data, with a correlation coefficient (R^2) up to 0.99. The model parameters are presented in Table 4.2-1. Maximum of cumulated hydrogen production (P) on glucose was 31 ± 12 ml_{H2}·g⁻¹ eq.initial COD on glucose, which was almost a third higher than on cellobiose and MCC, two polymers of glucose. The average maximum hydrogen production yields were also different between cellobiose and MCC with 23 and 12 ml_{H2}·g⁻¹ eq.initial COD, respectively. In accordance with the polymerisation degree, the hydrogen production rate (R_m) was negatively impacted for hexose-based substrates since 29 ± 12 ml_{H2}·g⁻¹ eq.initial COD·d⁻¹ was observed on glucose and 12 ± 1 and 1.5 ± 0.6 ml_{H2}·g⁻¹ eq.initial COD·d⁻¹ on cellobiose and cellulose, respectively. Lag phase (λ) was similarly impacted with 12 days for MCC but only 2 days for glucose and cellobiose.

Table 4.2-1: Kinetic parameters of hydrogen production in batch tests according to the initial substrate concentration determined from modified Gompertz equation with P the maximum cumulative hydrogen production ($\text{mlH}_2\cdot\text{g}^{-1}\text{eq initial COD}$), Rm the maximum hydrogen production rate ($\text{mlH}_2\cdot\text{g}^{-1}\text{eq initial COD}\cdot\text{d}^{-1}$), λ the lag phase (days) and R^2 the coefficient of determination of the model. Values correspond to the average of four replicates \pm standard deviation observed between these replicates. Values in % in the H_2 yield column correspond to the proportion of the maximum theoretical value suggested by Hawkes et al. (2007), i.e 2.5 $\text{molH}_2\cdot\text{mol}^{-1}$ degraded substrate in glucose equivalent.

Substrate	Gompertz equation parameter values				H_2 yield ($\text{mmolH}_2\cdot\text{g}^{-1}\text{eq. COD consumed}$) and % of the Hawkes maximum theoretical value
	P ($\text{mlH}_2\cdot\text{g}^{-1}\text{eq.COD initial}$)	Rm ($\text{mlH}_2\cdot\text{g}^{-1}\text{eq.COD initial}\cdot\text{d}^{-1}$)	λ (days)	R^2	
Glucose	31 \pm 12	29 \pm 12	1.9 \pm 0.2	0.999 \pm 0.001	1.7 \pm 0.6 (13 \pm 5 %)
Cellobiose	23 \pm 2	12 \pm 1	2.1 \pm 0.2	0.999 \pm 0.001	4.2 \pm 0.6 (32 \pm 4 %)
Cellulose	12 \pm 5	1.5 \pm 0.6	12.1 \pm 0.5	0.997 \pm 0.004	8 \pm 4 (62 \pm 31 %)
Arabinose	67 \pm 6	32 \pm 4	2.04 \pm 0.06	0.999 \pm 0.001	6.4 \pm 0.4 (49 \pm 3 %)
Xylose	94 \pm 17	40 \pm 11	2.4 \pm 0.3	0.999 \pm 0.001	6 \pm 1 (44 \pm 8 %)
Xylan	32 \pm 5	1 \pm 4	3.2 \pm 0.2	0.999 \pm 0.001	4.1 \pm 0.6 (31 \pm 5 %)
Straw	10 \pm 9	13 \pm 13	3 \pm 1	0.998 \pm 0.002	1.3 \pm 0.6 (10 \pm 4 %)

Consistently, several authors showed that hydrogen production mainly depends on the polymerisation degree of carbohydrates (Danilenko et al., 1993; Monlau et al., 2012a; Quéméneur et al., 2012b; Sambusiti et al., 2013). Indeed, a hydrolysis step of carbohydrate polymers is required to produce simpler molecules that can further enter into the microbial cells to be metabolized. This step impacts directly the bioprocess kinetics (Kumar et al., 2009). Indeed, the hydrolysis step requires energy in the forms of ATP and NADH equivalents that reduces final hydrogen production. Therefore, high hydrogen yields are usually observed in presence of high soluble sugars-content substrates as previously reported (Guo et al., 2014; Monlau et al., 2012b).

The modified Gompertz model parameters were also estimated for pentose-based substrates. The C5 substrates showed higher hydrogen yields than C6 substrates, when comparing sugars with equivalent polymerisation degrees. As an illustration, the fermentation of arabinose and xylose led to an increase of the P_{max} value of 2 and 3 times, respectively, when compared to glucose. The maximum hydrogen production rate Rm was also higher with 32 and 40 $\text{mlH}_2\cdot\text{g}^{-1}\text{eq.initial COD}\cdot\text{d}^{-1}$ for arabinose and xylose, respectively. With C5 substrates, the polymerisation degree had also a

negative effect on the overall hydrogen production performances, since lower P and R_m and higher λ were observed for xylan than with xylose.

Nonetheless, these results suggest that pentoses are preferred carbon sources for the production of hydrogen using this inoculum and under these experimental conditions. Similar observation was already made by Prakasham *et al.* (2009) who used a mixed inoculum issued from a buffalo dung compost, although the reported differences between hexose and pentose substrates were lower than in the present study. Mäkinen *et al.* (2012) also obtained the same trend with hot spring culture. All these observations suggest that metabolic pathways are strongly dependent on the origin of the inoculum especially when considering monomeric carbohydrates. Moreover, fermentative communities seem more favourable to produce hydrogen from C5 than from C6.

In the case of wheat straw, the complex structure of this material led to very poor hydrogen value which is highly consistent with the literature (Agbor *et al.*, 2011). Similarly to micro-crystalline cellulose, only 8% of the initial equivalent COD was converted with very low hydrogen accumulation.

When comparing the quantity of hydrogen produced per unit of COD converted during the process, i.e. hydrogen production yield (Table 3.2-1), it was concluded that more consumed COD was transformed into hydrogen for MCC and cellobiose than from glucose, with 8, 4.2 and 1.7 mmol_{H₂}·g⁻¹_{eq.COD consumed}, respectively. Even though the degradation was weaker on cellobiose and MCC than on glucose, metabolisms of the microflora converting the polymer seemed to be oriented to the production of hydrogen contrary to what observed on glucose.

In summary, hydrogen production correlated well with both the degree of polymerisation and the type of residual sugars (C5 or C6). When considering the relative amount of COD converted to H₂, cumulated hydrogen represented a COD fraction ranging from 11 to 2% only. This result should be compared to a theoretical value of 21% for mixed cultures, as suggested by Hawkes *et al.* (2007). Therefore, the other metabolites co-produced during fermentation have also to be considered.

4.2.2 Other fermentation metabolites

Soluble fermentation products were analysed at the end of the batch tests. The amounts of metabolites expressed in mg_{product eq. COD}·g⁻¹_{initial substrate eq. COD} are presented in Table 4.2-2. A principal component analysis (PCA) was carried out to illustrate the variability in metabolic pathways between the different substrates (Figure 4.2-2).

Table 4.2-2: Metabolites accumulated at the end of the fermentation tests (in $\text{mg}_{\text{product eq. COD}} \cdot \text{g}_{\text{initial substrate eq. COD}}^{-1}$.) Values correspond to the average of four replicates \pm standard deviation. Values in italic and in parenthesis correspond to the proportion (in %) of each product among all the metabolites produced in a same batch test \pm standard deviation.

Sample	Acetate	Butyrate	Isobutyrate	Propionate	Lactate	Ethanol	Hydrogen	COD converted (%)
Glucose	20 \pm 8 <i>(2.4\pm0.9)</i>	116 \pm 42 <i>(14\pm5)</i>	/	/	592 \pm 11 <i>(76\pm6)</i>	44 \pm 4 <i>(5.5\pm0.4)</i>	21 \pm 8 <i>(2.5\pm0.9)</i>	79 \pm 3
Cellobiose	11 \pm 4 <i>(5\pm2)</i>	11 \pm 11 <i>(5\pm5)</i>	/	/	165 \pm 12 <i>(73\pm2)</i>	23 \pm 3 <i>(11\pm1)</i>	15 \pm 2 <i>(6.8\pm0.7)</i>	23 \pm 1
MC-Cellulose	25 \pm 5 <i>(54\pm7)</i>	7 \pm 2 <i>(19\pm9)</i>	/	8 \pm 3 <i>(15\pm5)</i>	/	0.6 \pm 0.6 <i>(1\pm1)</i>	6 \pm 3 <i>(11\pm6)</i>	4.68 \pm 0.03
Arabinose	86 \pm 7 <i>(20\pm3)</i>	146 \pm 46 <i>(31\pm8)</i>	54 \pm 6 <i>(12.5\pm0.6)</i>	/	48 \pm 17 <i>(13\pm5)</i>	61 \pm 30 <i>(13\pm5)</i>	45 \pm 3 <i>(10.5\pm0.8)</i>	44 \pm 6
Xylose	118 \pm 14 <i>(19\pm2)</i>	247 \pm 82 <i>(34\pm10)</i>	35 \pm 18 <i>(5\pm2)</i>	72 \pm 35 <i>(14\pm7)</i>	6 \pm 6 <i>(0.9\pm0.9)</i>	104 \pm 16 <i>(18\pm4)</i>	62 \pm 11 <i>(9.6\pm0.7)</i>	64.4 \pm 0.9
Xylan	111 \pm 20 <i>(35\pm7)</i>	88 \pm 50 <i>(26\pm14)</i>	8 \pm 2 <i>(2.4\pm0.4)</i>	12 \pm 7 <i>(4\pm2)</i>	/	83 \pm 19 <i>(25\pm6)</i>	21 \pm 3 <i>(7\pm1)</i>	32 \pm 2
Wheat Straw	38 \pm 7 <i>(50\pm5)</i>	26 \pm 3 <i>(35\pm5)</i>	4 \pm 2 <i>(5\pm2)</i>	4.7 \pm 0.6 <i>(6.3\pm0.8)</i>	/	0.7 \pm 0.7 <i>(1\pm1)</i>	1.6 \pm 0.7 <i>(2\pm1)</i>	8 \pm 1

COD conversion was evaluated by COD mass balance according to metabolites concentrations. Because batch tests were stopped at the maximum of hydrogen production, the initial substrate COD was not necessarily fully converted at the end of the experiment. In particular, the percentage of COD converted at the final time decreased from 79 \pm 3 to 4.68 \pm 0.03 according to the polymerisation degree for the C6 substrates. Indeed, the compact structure of the micro-crystalline cellulose is an obstacle for hydrolytic enzymes to access substrate linkage. Complex substrates may not be fully converted at the end of the process as it was observed in the present study (Kumar et al., 2009).

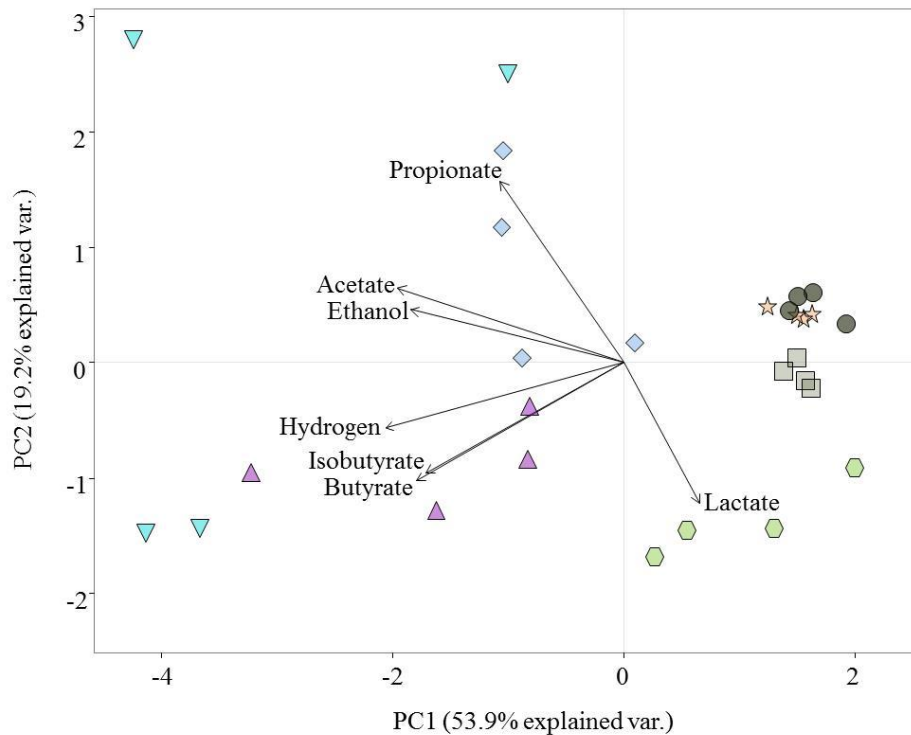


Figure 4.2-2: Principal component analysis done on metabolite production. Symbols correspond to each substrate, Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★).

Similarly to hexose substrates, the COD converted at the end of pentose fermentation was higher for the monomer than for the polymer supporting that the polymerisation degree also had an impact regardless the sugar unit.

The use of xylose and arabinose as different C5 isomers (on carbon 2) showed various conversion yields since only 44% of the initial COD was converted under the form of metabolites for arabinose against 64.4% for xylose. Conversion of arabinose and xylose implies the use of different enzymes. L-arabinose is first converted by via L-arabinose isomerase, L-ribulokinase and L-ribulose-5-P 4- epimerase to L-ribulose, L-ribulose-5- phosphate and D-xylulose-5-phosphate respectively, whereas D-xylose is directly transformed into D-xylulose by D-xylose isomerase (Bettiga et al., 2009). In conclusion, bacteria will have the ability to convert one pentose, the other or both, depending on their enzymatic potential.

Overall, the metabolic patterns appeared to be strongly linked to the type of carbohydrates (C6 or C5). Hexoses (glucose and cellobiose) were mainly converted into lactate (>70%). The hydrogen yield was likely linked to the production of butyrate as second main metabolite, as previously suggested by Guo *et al.* (2014). Acetate and ethanol represent less than 10% of all the

metabolites produced. The proportion of acetate and butyrate produced was higher for the polymers of glucose than for the monomer. This metabolic pattern favourable to the production of hydrogen might explain the high value of hydrogen production yield for cellobiose and MCC, as showed in Table 4.2-1.

For C5 fermentation (xylose and arabinose), metabolisms were slightly different since very low amounts of lactate accumulated for arabinose, and butyrate was the main metabolic pathway (30%). Consistently, hydrogen yield was higher than with hexoses. Similar results were observed in a previous study. Using hot spring culture as main fermentative microorganism source, Mäkinen *et al.* (2012) showed that lactate was preferentially produced during glucose conversion and pentoses were converted to butyrate and also formate, a metabolite unfound in the present study.

Ethanol and acetate were produced in higher quantity from C5 than from C6 (x10) and a new metabolite, isobutyrate, was identified. No information about isobutyrate production from pentose-based sugars was found in the literature. Nonetheless, the conversion into ethanol has been largely studied in the field of biofuel production. Even though yeast is the main microorganism studied for ethanol production, bacteria are also able to produce bio-ethanol (Bettiga *et al.*, 2009).

Additionally, identification of different by-products depending on the type of pentose sugar was observed. While lactate was more produced during arabinose conversion, propionate was specific of xylose and xylose-based polymers. Indeed, bacteria did not have the same capacity to convert the two pentoses as explained before, that in counterpart depends greatly of the microbial community composition

4.2.3 Strong selection in microbial communities

The differences in metabolic patterns observed for each substrate might be the consequence of different metabolic pathways within the same microbial community or due to the selection of specific bacterial communities. To resolve this, bacterial DNA was extracted from each sample and the V3 region of the RNA 16S ribosomal region was amplified and sequenced by high-throughput amplicon sequencing. Principal component analysis (PCA) was used to represent the variability between each final community (Figure 4.2-3). Data were first modified using a Hellinger transformation in order to decrease the weight of OTUs present in minority that could hide the main information.

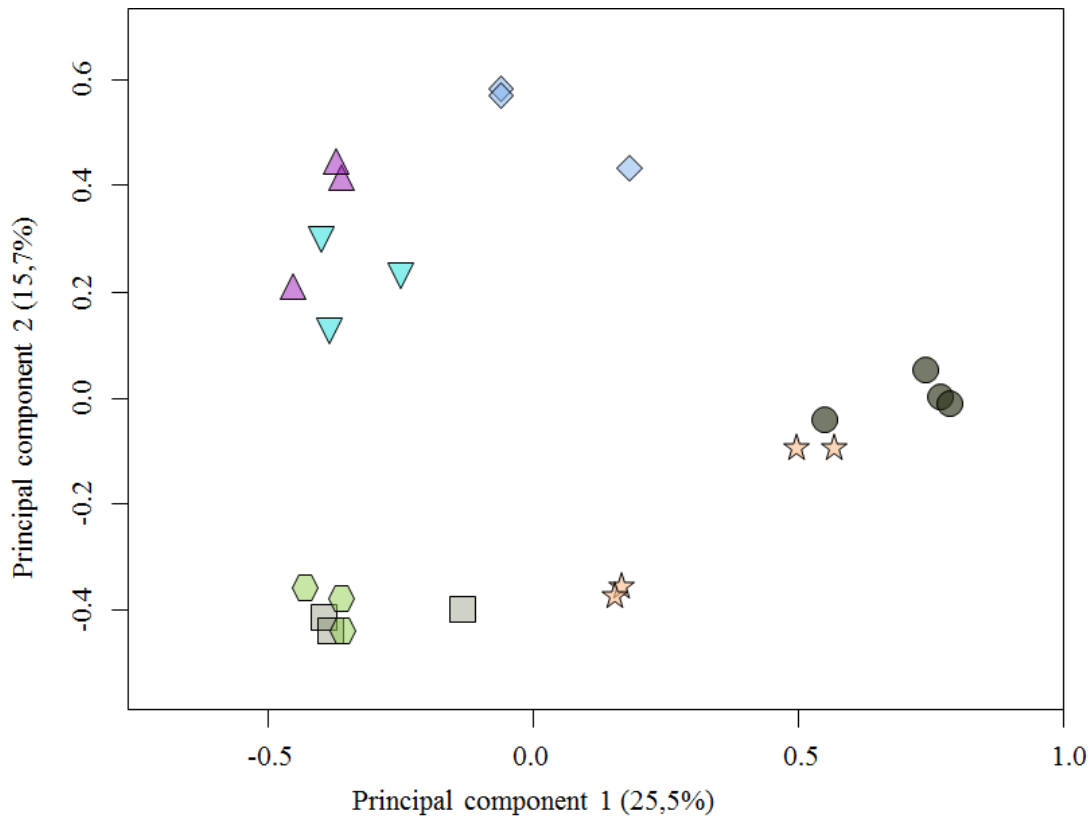


Figure 4.2-3: Principal component analysis (PCA) done on OTUs after Hellinger transformation. Symbols correspond to each substrate, Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★).

First, samples can be distinguished by the first principal component according to the polymerisation degree. Communities growing on monomers and dimers are located on the left side whereas polymers-based communities, including wheat straw, are on the right side. The second component was more related to the carbon content of the substrate. Similarly to the metabolic patterns, it was concluded that the final bacterial structure depended on both the polymerisation degree and the type of substrate.

The relative proportion of the major OTUs (relative abundance > 5%) found for each substrate are presented in Figure 4.2-4. Main OTUs were affiliated to the *Clostridiaceae*, *Bacillaceae* and *Peanibacillaceae* families where both pentose and hexose simple sugars were used as carbon sources (xylose, arabinose, glucose and cellobiose). These bacteria have been well described in mixed cultures fermentation. More particularly, members of the *Clostridiaceae* family have generally been associated to hydrogen production, while *Bacillaceae* and *Peanibacillaceae* have been described to be involved in lactic acid fermentation (Ghimire et al., 2015).

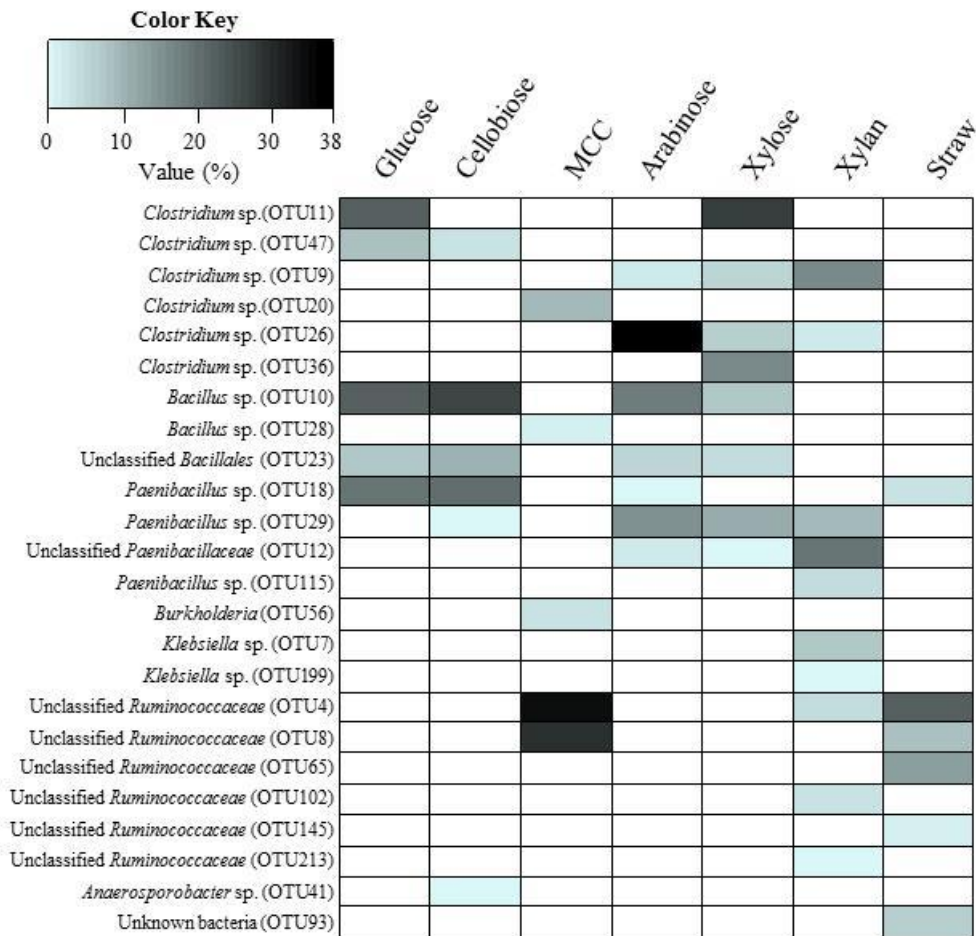


Figure 4.2-4: Major OTUs identified in reactors for each substrate (relative abundance >5%). White colour corresponds to absence of identified OTU, light blue a low relative abundance, and black relative abundance close to the maximum measured (38%)

Interestingly, these major OTUs were not found in the reactors containing complex substrates such as xylan, cellulose and wheat straw. Moreover, these OTUs were all affiliated to the *Ruminococcaceae* family. Bacteria belonging to this family are closely related to *Clostridium* sp. and are mainly microorganisms found in rumen. They have been reported as efficient degraders of complex biomass (Hung et al., 2011). Because *Ruminococcaceae* bacteria were not found in reactors fed with simple substrates, this suggests that *Clostridiaceae*, *Bacillaceae* and *Paenibacillaceae* were more competitive with regards to readily biodegradable sugars. The hydrolytic activity of the *Ruminococcaceae* confers to them an ecological advantage to grow on more complex organic materials.

Three proteobacteria were also identified in reactors fed with complex substrates. The OTU56 affiliated to the *Burkholderia* genus was present in MCC-based reactors. Similar bacteria were already described for their hydrolytic activity during complex organic biomass degradation (Mohana

et al., 2008). Their presence was often associated to the production of hydrogen (Porwal et al., 2008).

The OTU7 and OTU199 found in xylan-based reactors were both affiliated to the *Klebsiella* genus (Wong et al., 2014). These facultative anaerobes have been previously reported as efficient hydrogen producers. However, they exhibit a concomitant ethanol pathway which may explain the high proportion of ethanol (25±6%) found in these reactors (De Amorim et al., 2012).

Metabolites measured at the end of fermentation were fitted onto ordination performed with the main OTUs in order to estimate the correlation strength existing between microbial communities and associated metabolisms. Results show that acetate and lactate correlated better with the bacterial community structures than other metabolites (<0.1% of significance, Figure 4.2-5). Indeed, acetate production in C5-based sugar fed reactors was mostly explained by the presence of OTU9 and OTU26 affiliated to *Clostridium sp.* and OTU12 and OTU29 affiliated to *Paenibacillus sp.*. With lesser significance (<1% and 5%), the emergence of these bacteria also correlated with hydrogen, butyrate and ethanol production.

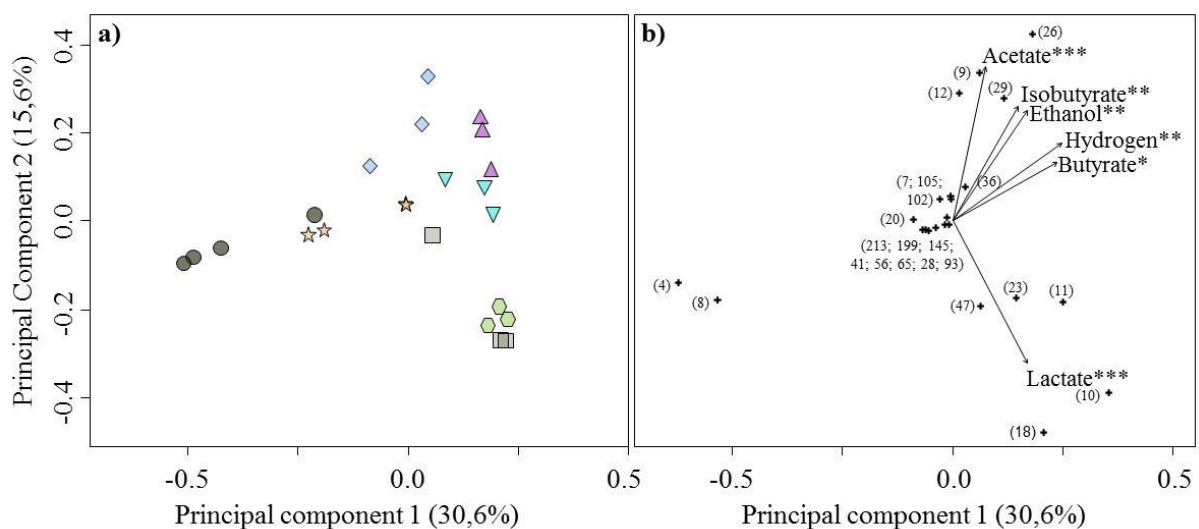


Figure 4.2-5: Principal component analysis (PCA) ordination plot of major bacteria composing the sample bacterial community (a) with metabolite variables as predictors onto the ordination (b) analysed by the *envfit* function (permutations 9999, R package *vegan*). Symbols correspond to substrates: Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★); and to number of Operation Taxonomic Unit (OTU, †). Stars following metabolite name represent the significance of the fit (***<0.001; **<0.01, *<0.05).

Lactate production was represented by OTU18 (*Paenibacillus sp.*) and OTU47 (*Clostridium sp.*) which were mainly identified in hexose-based reactors. Interestingly, OTU11 (*Clostridium sp.*), OTU9 and OTU20 affiliated to *Bacillus sp.* are located in the hexose substrate zone although they were present in both C6 and C5 based reactors. *Bacillus sp.* are facultative anaerobes particularly essential at the beginning of the fermentation since they favour the anaerobic conditions by consuming traces of residual oxygen (Huang et al., 2010). Their ability to grow on many carbohydrates may explain their presence in both hexose and pentose-based reactors (Stülke and Hillen, 2000).

In addition, few studies showed that the type of substrate can drive different bacterial community structures (Akutsu et al., 2009a; Cheng et al., 2011; Li and Chen, 2007; Qiu et al., 2016; Quéméneur et al., 2011a). Table 4.2-3 reports the hydrogen production yields reported in the literature using different bacterial cultures and various substrates. Overall, acetate and butyrate were the main metabolites observed, which was accompanied by higher hydrogen production yields than in the present work. Bacteria affiliated to *Clostridium sp.* were identified as the main microorganisms composing the mixed culture whereas in the present study, *Bacillus sp.* and *Paenibacillus sp.* represented a large part of the communities. These different structures of bacteria communities led in this case to the production of lactate as main metabolite in the case of hexose-based substrates.

Table 4.2-3: Hydrogen yields, metabolic patterns and main identified bacteria obtained from dark fermentation performed on different inocula and substrates in the literature. Units of hydrogen yield, H₂ column, correspond to a) mol_{H₂}·mol⁻¹_{substrate}, b) mmol_{H₂}·g⁻¹_{VS}, c) mmol_{H₂}·g⁻¹_{substrate}, d) mmol_{H₂}·g⁻¹_{TVS} and e) mmol_{H₂}·g⁻¹_{DCO}. Metabolites are expressed in % of COD converted and abbreviations correspond to Acetate (HAc), Butyrate (HBut); Caproate (HCap), Ethanol (EtOH), Lactate (HLac), Propionate (HPr), Formate (Hfor), Valerate (HVal) and Succinate (HSuc).

Inoculum	Substrate	H ₂	HAc	HBut	EtOH	HPr	HLac	HFor	HVal	HCap	HSuc	HiBut	Major Bacteria	References
anaerobically-digested sludge	fructose	1.84 ^a	34%	59%	6%	/	1%	/	/	0%	/	/	<i>C. sporogenes</i>	(Quéméneur et al., 2011a)
	glucose	1.79 ^a	33%	60%	6%	/	1%	/	/	0%	/	/	<i>C. sporogenes</i>	
	sucrose	1.67 ^a	86%	13%	1%	/	0%	/	/	0%	/	/	<i>C. sporogenes</i>	
	maltose	1.65 ^a	36%	58%	4%	/	0%	/	/	1%	/	/	<i>C. acetobutylicum</i>	
	cellobiose	1.56 ^a	35%	52%	9%	/	2%	/	/	1%	/	/	<i>C. cellulolyticum</i> ; <i>C. sporogenes</i> ; <i>C. Saccharobutylicum</i> ; <i>C. kluyveri</i> ; <i>C. acetobutylicum</i>	
	maltotriose	1.38 ^a	31%	48%	17%	/	3%	/	/	1%	/	/	<i>C. acetobutylicum</i>	
hot spring culture	xylose	0.7 ^a	25%	34%	5%	0%	1%	34%	/	/	/	/	<i>C. acetobutylicum</i> ; <i>C. tyrobutyricum</i>	(Mäkinen et al., 2012)
	arabinose	0.42 ^a	23%	31%	13%	0%	8%	25%	/	/	/	/	/	
seed sludge	xylose	2.24 ^a	17%	62%	13%	8%	/	/	/	/	/	/	<i>C. saccharobutylicum</i> ; <i>Clostridiales sp</i>	(Qiu et al., 2016)
anaerobic activated sludge	rice straw	6 ^d	67%	25%	7%	/	/	/	/	/	/	/	<i>Clostridium sp.</i>	(Cheng et al., 2011)
C. butyricum AS1.209	Corn straw	2.6 ^c	46%	28%	7%	19%	/	/	/	/	/	/	<i>C. butyricum</i> AS1.209 (%)	(Li and Chen, 2007)
Mesophilic digested sludge	Starch	6.1 ^e	15%	80%	2%	0%	0%	3%	/	/	0%	/	<i>C. acetobutylicum</i> ; <i>C. butyricum</i>	(Akutsu et al., 2009a)
	Soybean meal	5.6 ^e	27%	40%	31%	0%	0%	2%	/	/	0%	/	<i>C. acetobutylicum</i> ; <i>C. butyricum</i>	
Kitchen Waste		5.1 ^e	20%	71%	2%	0%	0%	6%	/	/	1%	/	<i>C. paraputrificum</i> ; <i>Citrobacter freufii</i>	
Cattle Manure		4.6 ^e	23%	49%	22%	0%	0%	7%	/	/	0%	/	<i>C. acetobutylicum</i> ; <i>C. butyricum</i>	
Activated Sludge		3.5 ^e	36%	5%	54%	2%	0%	3%	/	/	0%	/	<i>Bacteroides eggerthii</i> ; <i>C. pasteurianum</i>	

Inoculum	Substrate	H ₂	HAc	HBut	EtOH	HPr	HLac	HFor	HVal	HCap	HSuc	HiBut	Major Bacteria	References
Thermophilic Digested Sludge Soil		2.2 ^e	42%	7%	51%	0%	0%	0%	/	/	0%	/	<i>Clostridium</i> sp.;	
		2 ^e	31%	2%	51%	10%	0%	2%	/	/	4%	/	<i>Bacillus</i> sp. <i>Bacteroides</i> <i>eggerthii</i>	
Thermophilic Acidogenic Sludge		0.7 ^e	22%	0%	66%	0%	1%	4%	/	/	7%	/	<i>Bacteroides</i> sp.; <i>C.</i> <i>aminovalepticum</i>	
Manure anaerobic digestate	Glucose	0.60 ^a	2.4%	14%	5.5%	/	76%	/	/	/	/	/	<i>Clostridium</i> sp.;	This study
	Cellobiose	0.36 ^a	5%	5%	11%	/	73%	/	/	/	/	/	<i>Bacillus</i> sp. <i>Paenibacillus</i> sp.	
	MCC	6 ^e	54%	19%	1%	8%	/	/	/	/	/	/	<i>Ruminococcaceae</i>	
	Arabinose	0.45 ^a	20%	31%	13%	/	13%	/	/	/	/	12.5%	<i>Clostridium</i> sp.;	
	Xylose	0.62 ^a	19%	34%	18%	14%	0.9%	/	/	/	/	5%	<i>Bacillus</i> sp. <i>Paenibacillus</i> sp.	
	Xylan	21 ^e	35%	26%	25%	4%	/	/	/	/	/	2.4%	<i>Ruminococcaceae</i> ;	
	Wheat straw	1.6 ^e	50%	35%	1%	6.3%	/	/	/	/	/	5%	<i>Clostridium</i> sp.;	
													<i>Bacillus</i> sp. <i>Paenibacillus</i> sp. <i>Ruminococcaceae</i>	

Depending on the residues used, bacterial community developed different structures at the end of the fermentation that drove the metabolism towards lactate or acetate/butyrate production. Selection of bacteria represents an ecological advantage which favours the development of microorganisms on a specific substrate. Such sensitivity of bacterial communities to substrate composition should be strongly considered when using continuous systems (Bakonyi et al., 2014). Finely controlling the substrate input could be a mean of action to maintain a community structure within the reactor and, thus, the performance stability for hydrogen production. This could also lead to improve the biomass conversion yield by selecting specific bacteria able to degrade a complex substrate. The use of biological strategies to increase complex substrates into biohydrogen has already been performed in dark fermentation (Kotay and Das, 2009; Kuo et al., 2012). By adding keystone species in the medium as those identified in this study, it could therefore be possible to improve the production of hydrogen from organic residues without expensive and not environmental friendly pretreatment, as generally performed to deconstruct lignocellulosic materials.

4.2.4 Conclusion

In this chapter, this first experiment shows that the production of hydrogen was closely linked to the sugar type (pentose or hexose) and the polymerisation degree. The inoculum produced less hydrogen on hexose- than on pentose-based substrates. The low amount of hydrogen was explained by a specific selection of lactate-producing bacteria during cultivation. The selected bacteria were affiliated to the genera *Paenibacillus sp.* and *Bacillus sp.* On pentose-based substrates, the amounts of accumulated hydrogen were five-fold higher than on hexoses, with specific bacterial community structures producing acetate and butyrate as main metabolites. However, low hydrogen amounts accumulated from complex sugars (cellulose, xylan and wheat straw). Interestingly, the presence of *Ruminococcaceae sp.* on polymeric sugars (cellulose, xylan and wheat straw) suggests that these bacteria played an important role in the hydrolytic activity.

However, the structure of the initial bacterial community could affect these preliminary results. Such impact was evaluated using the same inoculum but after a storage period before dark fermentation. Results are described in the next section.

4.3 Impact of substrate composition on dark fermentation performed by stored digested manure inoculum (StM)

4.3.1 Impact of inoculum storage on hydrogen and other metabolites production.

As explained before, the same experiment was performed using the same inoculum but after a storage period of two months at 35°C with no feeding. Similarly to the fresh manure (FM) inoculum, hydrogen production was measured every eight hours and the experiment was stopped once a plateau was reached. No methane production was observed, validating the efficiency of heat shock pretreatment.

As a result, the fermentation time was 3.1 ± 0.2 days for arabinose, xylose and glucose, 5 ± 1 days for cellobiose and 8.7 days for xylan and wheat straw. Compared to the FM inoculum, the maximum of hydrogen production was reached faster on all substrates with the stored inoculum, with the exception of MCC for which no hydrogen was detected during the course of fermentation.

The kinetic parameters were estimated by fitting a Gompertz model to the hydrogen production data and results were compared to those obtained during the first experiment (Table 4.3-1). Coefficient of correlation is always higher than 0.99 (data not shown) which confirms a good fitting of the model. As main result, it the Gompertz parameters vary greatly according to the type of inoculum, fresh or stored.

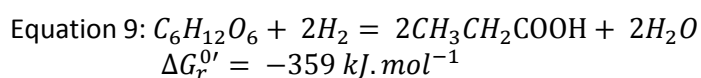
Table 4.3-1: Modified Gompertz parameters calculated for each substrate and for the two inocula, before (italic, FM) and after storage (bold underline, StM), with P the maximum cumulative hydrogen production ($\text{mlH}_2 \cdot \text{g}^{-1} \text{eq.COD initial}$), Rm the maximum hydrogen production rate ($\text{mlH}_2 \cdot \text{g}^{-1} \text{eq.COD initial} \cdot \text{d}^{-1}$) and λ the lag phase (days). Values correspond to the average of four replicates \pm standard deviation observed between these replicates.

Substrate	Gompertz equation parameter values						H ₂ yield ($\text{mmolH}_2 \cdot \text{g}^{-1} \text{eq. COD consumed}$)	
	P_{max} ($\text{mlH}_2 \cdot \text{g}^{-1} \text{eq.COD initial}$)		Rm ($\text{mlH}_2 \cdot \text{g}^{-1} \text{eq.COD initial} \cdot \text{d}^{-1}$)		λ (days)			
Glucose	<i>32±12</i>	<u>27±6</u>	<i>29±11</i>	<u>35±12</u>	<i>1.9±0.2</i>	<u>1.1±0.2</u>	<i>1.7±0.6</i>	<u>2.2±0.5</u>
Cellobiose	<i>23±2</i>	<u>20±6</u>	<i>12±1</i>	<u>10±3</u>	<i>2.1±0.2</i>	<u>1.4±0.5</u>	<i>4.1±0.6</i>	<u>1.1±0.3</u>
Cellulose	<i>12±5</i>	<u>0</u>	<i>1.5±0.6</i>	<u>0</u>	<i>12.1±0.5</i>	<u>0</u>	<i>8±4</i>	<u>0</u>
Arabinose	<i>67±6</i>	<u>19±8</u>	<i>32±4</i>	<u>17±5</u>	<i>2.04±0.06</i>	<u>1.2±0.1</u>	<i>6.4±0.4</i>	<u>1.4±0.4</u>
Xylose	<i>94±17</i>	<u>19±6</u>	<i>40±11</i>	<u>20±6</u>	<i>2.4±0.3</i>	<u>1.0±0.2</u>	<i>6±1</i>	<u>1.7±0.5</u>
Xylan	<i>32±5</i>	<u>17±12</u>	<i>11±3</i>	<u>10±7</u>	<i>3.2±0.2</i>	<u>2.4±0.2</u>	<i>4.1±0.6</i>	<u>1.2±0.9</u>
Straw	<i>10±9</i>	<u>0.6±0.2</u>	<i>13±13</i>	<u>0.4±0.1</u>	<i>3±1</i>	<u>1.88±0.02</u>	<i>1.3±0.6</i>	<u>0.7±0.3</u>

For simple hexose-based substrates, i.e. cellobiose and glucose, Gompertz parameters were similar whatever the inoculum. Only the lag phase was reduced when using the stored inoculum since the production of hydrogen started at about 2 days for FM culture whereas only 1.1 and 1.4 days on glucose and cellobiose, respectively were required for StM inoculum. No accumulation of hydrogen in the head space of MCC-based reactor was observed during the time of the experiment.

It can be noticed that the H₂ production yield of glucose was 23% higher after than before storage, with a simultaneous reduction of substrate conversion. Indeed, only 52±13% of glucose equivalent COD were converted into gas and metabolites for the inoculum used after storage, against 79±3% (Figure 4.3-1). This apparent decrease in substrate conversion is linked to the fact that the plateau of hydrogen production was reached faster in the case of StM inoculum than FM. At this time, the substrate was apparently converted in metabolites at lower amounts than with the FM inoculum.

Interestingly, the metabolic patterns observed during the degradation of glucose and cellobiose were more favourable to the production of hydrogen in the case of StM culture than for FM inoculum. Indeed, acetate and butyrate became the main metabolites that accumulated in reactors whereas the proportion of lactate decreased of 78% and 88% for glucose and cellobiose, respectively. More precisely, this shift in metabolic patterns towards the production of acetate and butyrate increased only the hydrogen production yield in the case of glucose but not for cellobiose. Since more acetate was produced than butyrate, it can be suggested that the homoacetogenic activity appeared in cellobiose-fed reactors. Indeed, it has been previously reported that some bacteria affiliated to the *Clostridiales* order could support homoacetogenesis during stress conditions (Saady, 2013). In the present study, such stress could result from the storage period without any feeding during two months. Indeed, substrate starvation can force bacteria to change their metabolic activity in order to survive (Link et al., 2015). By performing homoacetogenesis, bacteria can recycle hydrogen to continue their growth in case of depletion of reduced carbon (Yan et al., 2014). In the case of cellobiose, production of propionate can also explain the low accumulation of hydrogen. Indeed, the production of this metabolite occurs with the consumption of two moles of hydrogen as demonstrated on Equation 9 (Latrille et al., 2011).



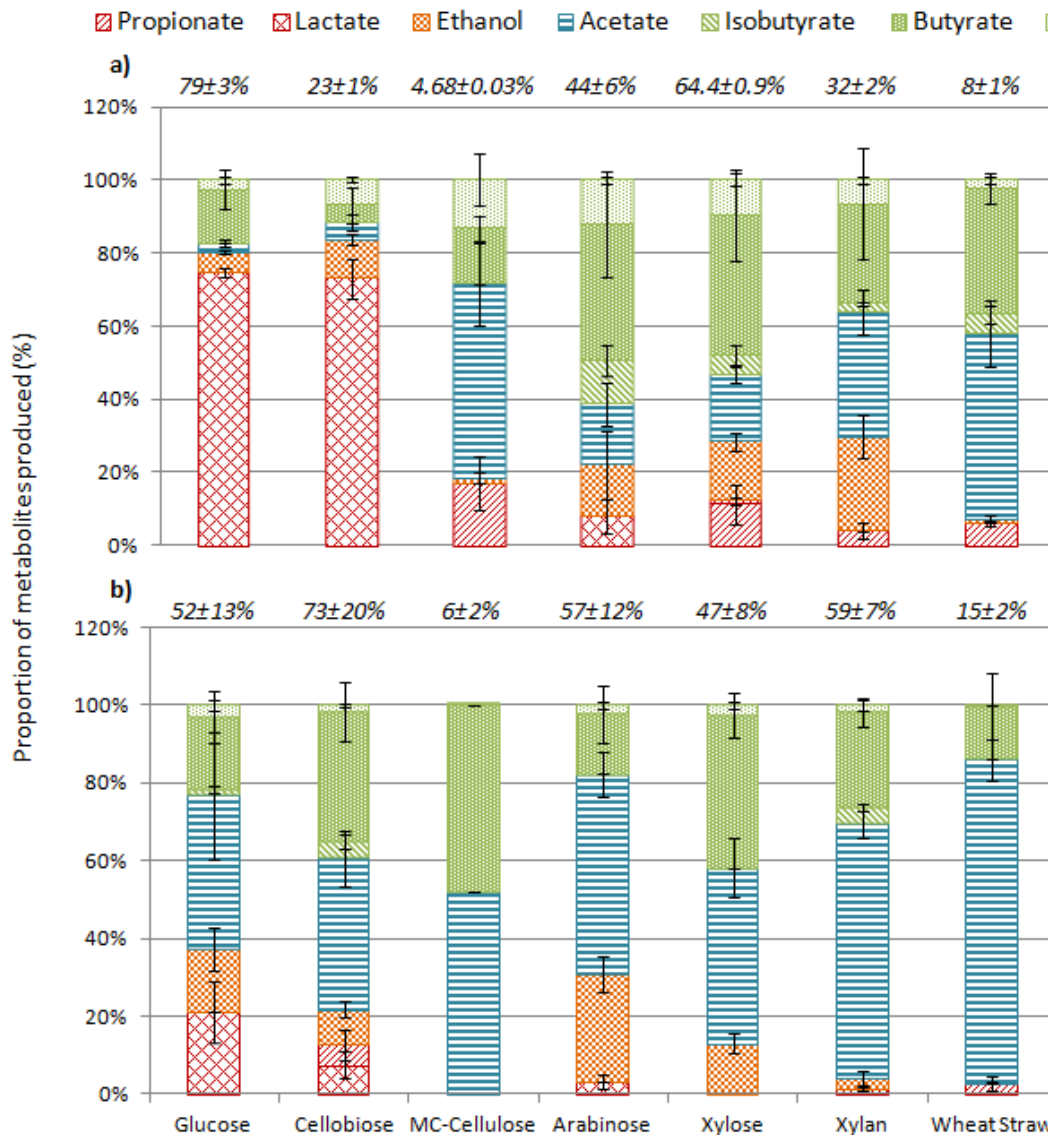


Figure 4.3-1: Metabolites patterns developed for each substrate by a) the inoculum without storage and b) the stored inoculum in $g_{ed. COD} \cdot 100g_{initial substrate eq. COD}$. Error bars represent the standard deviations calculated between the four replicates. Values above each bar correspond to the percentage of COD converted into metabolites at the end of the experiments \pm standard deviation observed between each replicate.

In the case of MCC, no hydrogen accumulation was observed during the time of the experiment with the StM inoculum. Nevertheless, the proportion of COD recovered under the form of metabolites was similar when using both StM and FM inoculum. The exclusive acetate/butyrate metabolism suggests that the hydrogen potentially produced with butyrate was immediately reused to produce acetate, which is consistent with the supposed homoacetogenic activity, as discussed above.

The storage period also modified the metabolic pattern developed during the conversion of pentose-based substrates. Whereas arabinose and xylose were preferential substrates of FM

inoculum to produce hydrogen, production yield obtained in the case of StM cultures was lower than on glucose, with 1.7 and 1.2 $\text{mmol}_{\text{H}_2}\cdot\text{g}^{-1}_{\text{COD consumed}}$, respectively (Table 4.3-1). It also resulted in a decrease of more than 72% of the P_{max} value whereas R_m was twice higher when using StM than FM. Such change was also observed for xylan for which hydrogen production yield dropped from 4.1 to 1.2 $\text{mmol}_{\text{H}_2}\cdot\text{g}^{-1}_{\text{COD consumed}}$. As observed for hexose-based substrates, these low productions of hydrogen were accompanied with the production of acetate as main metabolite (Figure 4.3-1). Here, acetate represented 52%, 45%, and 65% of all the metabolites produced during the conversion of arabinose, xylose and xylan respectively, while butyrate was mainly produced by the bacterial community issued from the FM inoculum. Same observation was done on the model lignocellulosic substrate, the wheat straw, for which 15% on the initial COD was converted to 86% of acetate.

Although the storage seems to negatively affect the production of hydrogen by inducing a stress and favouring the homoacetogenic activity, the percentage of the initial COD converted into metabolites at the end of the experiment increased for polymeric substrates. Whereas only 23%, 4.68%, 32% and 8% of the initial COD of cellobiose, MCC, xylan and wheat straw were respectively recovered under the form of metabolites when using the FM culture, these values increased to 68%, 22%, 46%, and 47%, respectively, in a shorter experimental time. This phenomenon may reflect a development of more efficient hydrolytic activity in the stored inoculum than in the fresh one.

Such modification of the fermentation activity observed between the two inocula can be explained by the selection of different bacterial communities or by the development of different metabolisms in a similar bacterial community. To estimate the impact of the storage period on the bacterial community structure selected after the dark fermentation, molecular biology analyses were performed on the different samples.

4.3.2 Bacterial communities structure developed in stored inoculum

Similarly to the experiment performed with the FM inoculum, the RNA 16S V3 ribosomal region of the bacteria community at the end of fermentation was sequenced by high-throughput amplicon sequencing. To compare the data sets of the two experiments, all sequences were aligned together before analysis.

Dissimilarities between each selected bacterial communities from StM culture were evaluated using PCA after data transformation by Hellinger method. Result of the first two principal components (PC) was represented on the ordination in Figure 4.3-2.

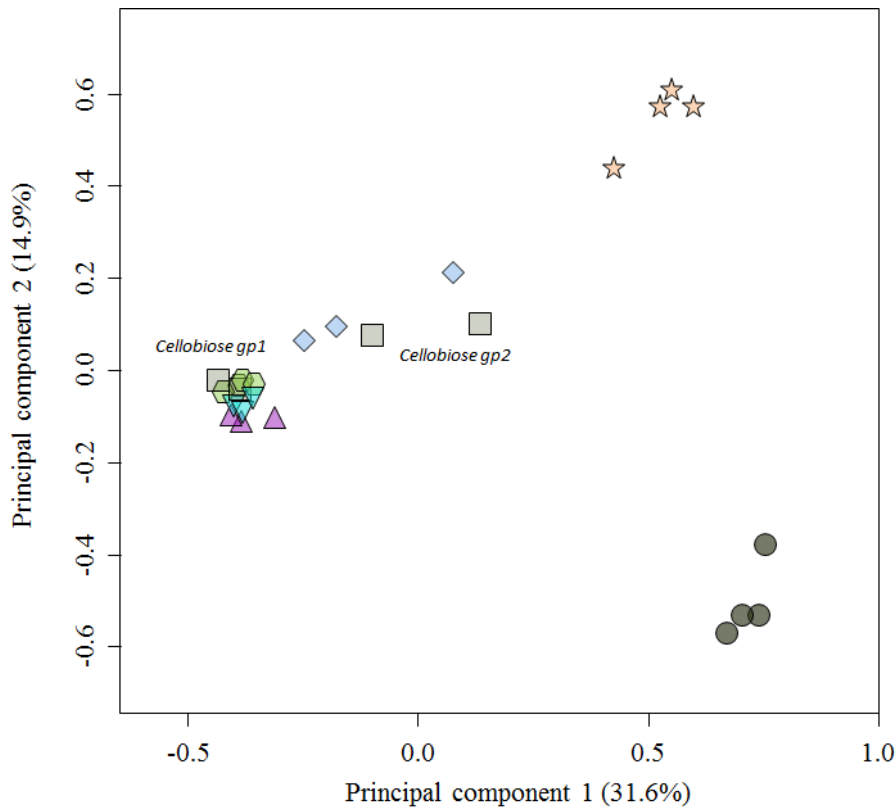


Figure 4.3-2: PCA realised on OTUs after Hellinger transformation. Symbols correspond to each substrate, Glucose (●), Cellobiose (■), Microcrystalline cellulose (●), Arabinose (▲), Xylose (▼), Xylan (◆) and Wheat Straw (★).

It can be observed that bacterial community composing each sample can be split into different groups according to the type of substrate along the two first PCA components. Simple carbohydrates are separated from polymeric sugars along the first PC. Cellobiose- and xylan-based reactors are placed between simple and complex substrates, suggesting that community developed in reactors fed with these two sugars share similarities with both communities selected on simple and complex carbohydrates. The second PC separates reactors fed with MCC than those fed with wheat straw, suggesting that differences exist between bacterial community structures selected from these two substrates. However, reactors fed with simple carbohydrates form one tight cluster on the ordination. This result suggests that bacterial community structure selected during the fermentation of glucose, arabinose, xylose and two samples of cellobiose are highly similar. Consequently, the specific selection of bacterial community according to the molecular composition of carbohydrate observed in the previous chapter was not anymore obtained using manure digestate as inoculum after two months of storage.

To identified which bacteria that emerged using StM inoculum, relative abundance of the major OTUs (>5%) identified for each substrate was represented under the form of heatmap in Figure

4.3-3. In the case of simple substrates, three main OTUs affiliated to *Clostridium* sp. (OTU17; OTU9) and unclassified *Paenibacillaceae* (OTU12) were found in glucose-, arabinose- and xylose-fed reactors, representing 42, 38 and 61% of the total sequenced OTUs, respectively. Two others sequences affiliated to the *Paenibacillaceae* family (OTU45 and OTU70) were also found in these three reactors, at a lower extent. Their total relative abundance was higher in arabinose (24.5%) than in glucose and xylose-based reactors (5.6 and 8.6%, respectively).

As previously observed, metabolic patterns were similar during the fermentation of glucose, arabinose and xylose using StM inoculum, in relation to the selection of a common bacterial community structure. In contrast with the FM culture for which the bacterial community was selected according to the type of carbohydrates, it is possible that the storage period pre-selected a community that was able to grow on simple sugar regardless its type which confers an advantage when growing on complex substrate.

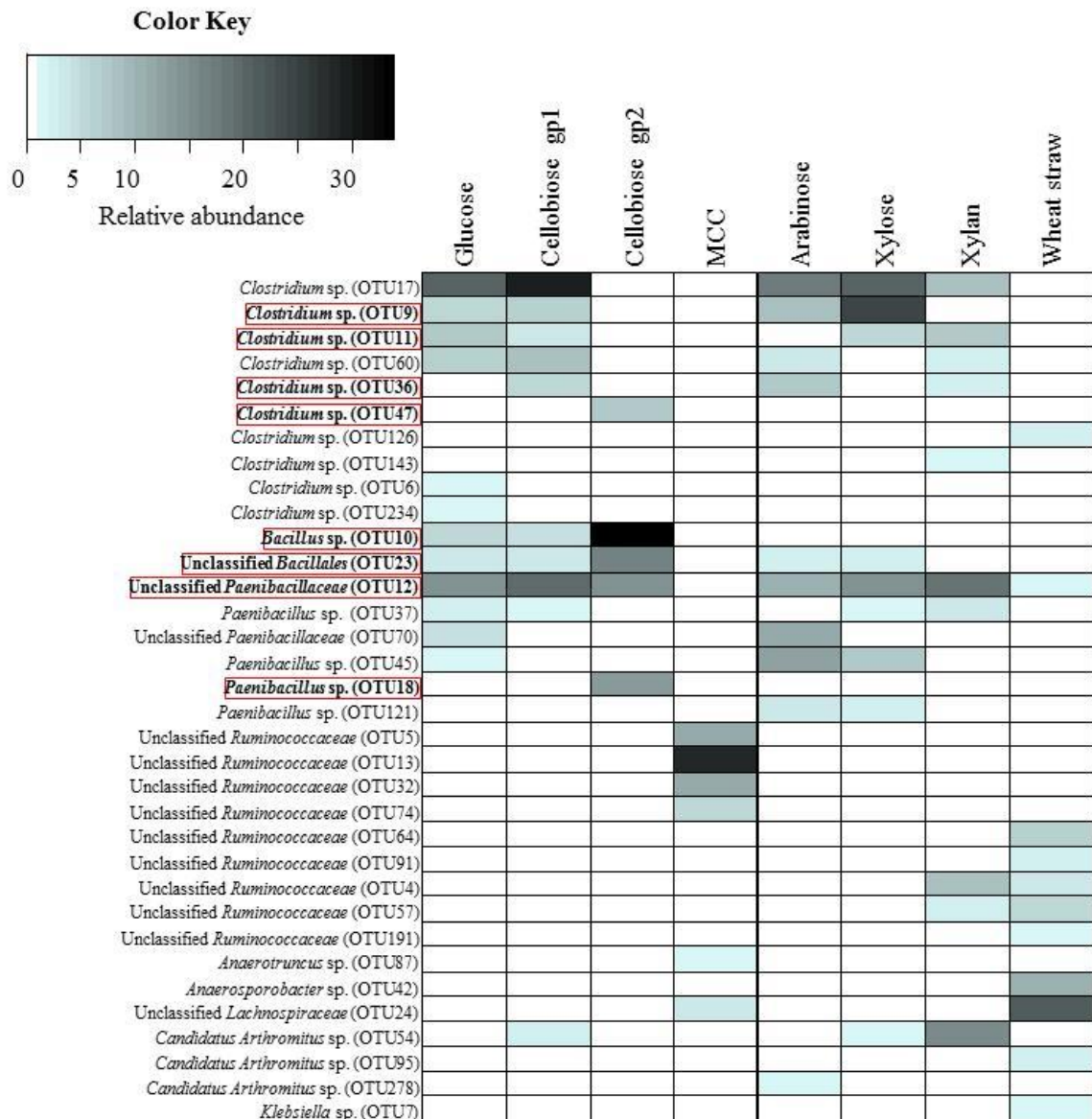


Figure 4.3-3: Heatmap representing the average of relative abundance of the major OTUs identified at the end of the fermentation of each sugar. White colour corresponds to non-identification of the OTU, light blue and black to low and high relative abundance, respectively (max=35%). OTUs that were identified in bacterial community of both FM and StM inocula are framed in red.

Communities grown on cellobiose and xylan, dimer and polymer of glucose and xylose respectively, were statically grouped with monomeric carbohydrates, but were more distant from these communities, as shown on Figure 4.3-2. For cellobiose-based reactors, two samples were closer to simple sugars than the two others. Because of this, the two groups are represented in two columns on Figure 4.3-3 (Cellobiose gp1 and Cellobiose gp2). On the heatmap representation, a common main OTUs was identified in both simple sugar-based reactors and those containing cellobiose and xylan, i.e. *Clostridium* sp. (OTU17) and unclassified *Paenibacillaceae* (OTU12). However, the OTU17 was not identified in the second group of cellobiose-based reactors, and other

sequences affiliated to *Clostridium* sp. and closely related were found in cellobiose gp1, e.g. OTU9; OTU11 and OTU60. In contrast, the three major OTUs OTU10, OTU23 and OTU12, were affiliated to the *Bacillales* order with 66.7% of total relative abundance. Such difference can contribute to the distance observed between the second group of cellobiose samples and the communities of simple sugar.

In the case of the communities developed in xylan-based reactors, the difference of bacterial community structure appeared with the identification of unclassified *Ruminococcaceae* (OTU4) and *Candidatus Arthromitus* (OTU54). Microorganisms affiliated to these families and genus have been described as non-pathogenic intestine bacteria. While *Ruminococcaceae* bacteria are known to perform hydrolytic activity, little information is available on *Candidatus Arthromitus*. They are segmented filamentous bacteria (SFB) recently affiliated to *Lachnospiraceae* family that sporulate and are attached to the gut cell walls of many animals, e.g. mammals or insects (Snel et al., 1995; Thompson et al., 2012). They were identified in wood feeding termites suggesting that they are able to degrade complex substrates, such as xylan (Margulis et al., 1990).

The high distance separating the bacterial communities developed in these previous substrates from those identified in wheat straw and MCC-based reactors showed that the bacterial community structure was different when complex substrates are considered. Indeed, the common OTUs affiliated to *Bacillales* order and *Clostridiaceae* family were not anymore identified in these communities. Instead of these bacteria, bacterial community developed on MCC was composed of microorganisms affiliated to *Ruminococcaceae* family (OTU5, OTU13, OTU32, OTU74) which represented 59% of the total community.

In the case of the community selected on wheat straw, some members of the *Ruminococcaceae* family were also identified, with different sequences, i.e. OTU4, OTU57, OTU64, OTU91 and OTU191. Their relative abundance was lower than on MCC, since these five OTUs represented only 22% of the total abundance. Two OTUs affiliated to the *Lachnospiraceae* family were identified on wheat straw, *Anaerosporebacter* sp. (42) and unclassified *Lachnospiraceae* (OTU24), representing together 33% of relative abundance. Bacteria affiliated to this family are also intestine bacteria particularly present in human colon (Duncan et al., 2007). They have been reported to produce cellulosomes, multi-enzyme complexes that hydrolyse lignocellulosic polymers (Thomas et al., 2014). That could explain their specific selection on complex substrates. As these bacteria were only identified in communities degrading xylan and wheat straw and not MCC, these strains might be specific of pentose-based substrates. Such selection of hydrolytic bacteria in polymer-based reactors

is coherent with the increase of COD converted into metabolites as presented in the previous section.

Hence, comparing to the previous experiment, it appears that when using manure anaerobic digestate after storage, bacterial communities are not anymore selected according to the monomeric composition of the initial carbon source but rather on the degree of complexity of the initial biomass. Further analyses were performed to see which of substrate type or inoculum has the highest impact on the final selection of the microbial community.

4.3.3 Comparison of bacterial community culture selected on fresh and stored inocula for the different substrates.

As sequences identified in FM and StM inocula were aligned together, it was possible to perform statistical analysis to investigate the differences existing between the structures of the bacterial community at the end of fermentation. A PCA was used to represent the variability between communities when using inocula with and without storage. The PCA result is represented on the ordination in Figure 4.3-4. According to the ordination, samples can be divided into three groups according to the substrate complexity and the inoculum used.

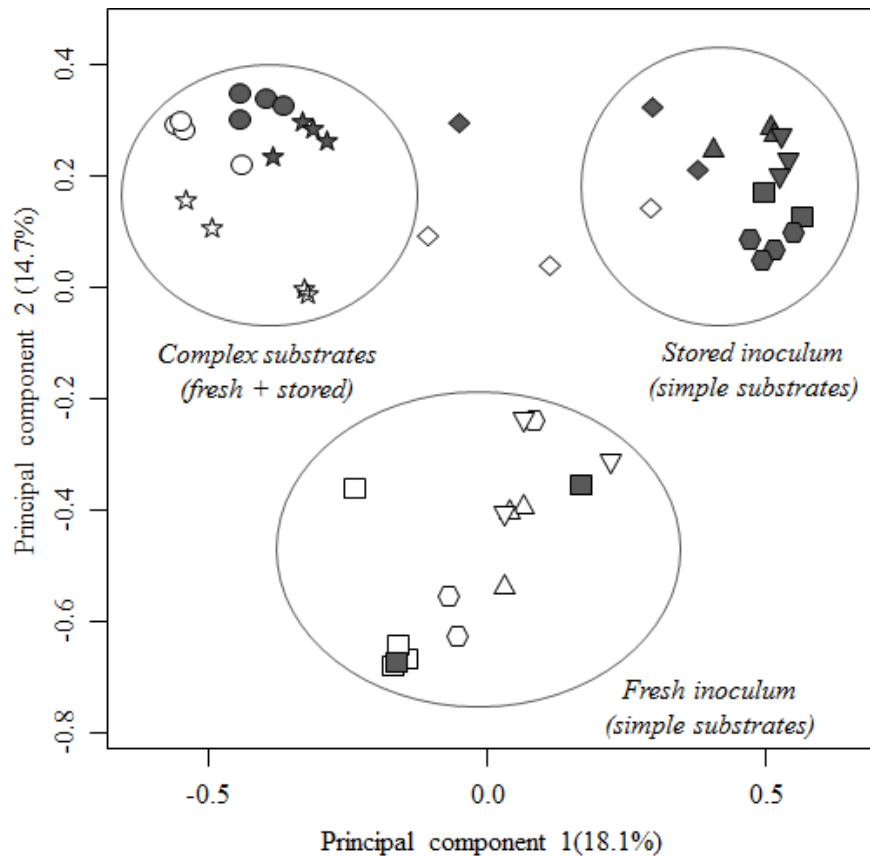


Figure 4.3-4: PCA performed with sequencing data of both fresh (white symbols) and stored inocula (black symbols). The forms of symbols correspond to the different substrates used for the fermentation, i.e. glucose (\hexagon), cellobiose (\square), MCC (\circ), arabinose (\triangle), xylose (∇), xylan (\diamond), and wheat straw (\star).

All bacterial communities developed from FM inoculum on simple substrates are pooled together at the bottom of the ordination whereas those composing simple substrates-based reactor from StM culture are placed on the top-right. This separation of sample based on community structure suggests that on simple substrates the type of initial inoculum had a higher impact on the final community structure than on the type of carbohydrate. Even considering that anaerobic degradation of sugars depended mainly on the type of carbohydrates, most of microorganisms are able to ferment glucose, arabinose and xylose. Therefore, bacterial selection was mainly dependent on their presence in the initial culture.

Interestingly, it can be noticed that distances between communities growing on hexose and pentose-based substrates inside the fresh inoculum group are higher than those observed in the stored one. Indeed, difference of community structure was noticed from microbial analysis performed on these samples (4.2).

Different behaviours can be observed on complex substrates, i.e. MCC, xylan and wheat straw. The distances between bacterial communities growing on these substrates were lower than those of simple substrates, which were pooled together at the top-left of the ordination, regardless the type of inoculum. As some sequences were identified in both communities developed on simple and complex substrates and were also observed in xylan-based reactors, samples of this complex substrate were placed between groups of simple substrate (stored manure) and complex biomass. This observation suggests that, contrary to simple substrates, a specific bacterial community structure was selected according to the inoculum to convert the complex biomass. As observed in the previous section, these bacteria were affiliated to *Ruminococcaceae* and *Lachnospiraceae* families, known to perform efficient hydrolytic activity as required to convert this type of biomass.

Overall, according to these results, the structure of the bacterial community after fermentation of simple substrates is linked to the type of inoculum used, regardless the composition of the substrate, which will lead to the variable accumulation of hydrogen, depending on the metabolic pattern developed. In the case of complex substrates, the bacterial community tends to form a similar community based on the presence of *Ruminococcaceae*. This new observation can be determinant when setting up an experiment. Indeed, when considering complex substrates, the organisation of the bacterial community to a common structure allows using any inoculum whatever its origin. This is an economical advantage since the user can choose any type of mixed culture. However, in case of simple substrate, the present work shows that the choice of the inoculum has to be strongly considered before running dark fermentation since the amount of hydrogen will mainly depend on its type.

4.4 Conclusion

During this chapter, it has been shown that the type of substrate had an influence on both metabolic patterns and the structure of the final bacterial community.

When using an inoculum freshly sampled from manure anaerobic digestate as source of fermentative bacteria, the final bacterial community presented different structures according to the type of substrate, i.e. pentose- or hexose-based sugar or degree of polymerisation. It resulted in the development of different metabolic patterns with the accumulation of various amounts of hydrogen. The maximum of hydrogen production yield was obtained on arabinose with acetate/butyrate metabolism developed by the mixed culture in which *Clostridium* sp. were present in major quantity. Low hydrogen production in glucose-based reactors was explained by the apparition of a lactate-based metabolism, which was carried by bacterial communities rich in *Bacillales*. The low quantity of

hydrogen measured in reactors fed with complex substrates, i.e. xylan, MCC and wheat straw, was due to the low conversion of biomass once the maximum of hydrogen production was reached. Hence these substrates needed a first hydrolysis step in order to release monomeric sugars, which limited the production of gas. Nonetheless, a hydrolytic community mainly composed of members from *Ruminococcaceae* family was selected during the fermentation of these substrates.

The use of the same inoculum after two months of starvation led to different conclusions, showing the high impact of the initial community of fermentation behaviour. Contrary to the previous experiment, metabolic patterns developed during the fermentation on the same substrates were similar for all substrates. Acetate was the main metabolite produced, followed by butyrate. Because low amounts of hydrogen were measured, a homoacetogenic activity was suspected. In the case of reactors fed with simple carbohydrate, i.e. glucose, arabinose, xylose, and sometimes cellobiose, this common metabolism was the result of the activity performed by a similar bacterial community, composed of bacteria from both the *Clostridiaceae* family and *Bacillales* order. In the case of complex substrates, i.e. xylan, MCC and wheat straw, other bacteria affiliated to *Ruminococcaceae* family were identified in majority in the selected community, as it was observed when using the fresh inoculum. Moreover, bacteria affiliated to another family able to produce hydrolytic enzymes, i.e. *Lachnospiraceae*, were also identified.

Hence, by comparing bacterial communities selected from the two types of inoculum, it was observed that when using complex substrates, the final bacterial communities converged on a similar structure to hydrolyse the material. In contrast, the final structure selected from the fermentation of simple substrates appeared to be inoculum dependant. Such impact of the inoculum community structure may have drastic consequence of the production of hydrogen as observed in this work, and knowing the key indicators in inoculum that could favour the H₂ production is of high importance. Next chapter will focussed on the influence of the initial bacterial structure of inocula on the process of dark fermentation.

Chapter 4. Relationships between initial and final bacterial community structures, and metabolisms identified after dark fermentation

4.1 Context and objectives

The combination of all fermentative bacteria, forming a mixed culture, offers a global fermentative pattern that can lead to the production of hydrogen under very specific conditions (Latrille et al., 2011). Depending on the bacteria composing the microbial community, the hydrogen production performances and associated metabolic patterns can vary from one inoculum to another (Akutsu et al., 2009a). *Clostridiales* bacteria are generally reported as hydrogen-producing microorganisms whereas most of bacteria from the *Bacillales* order are generally known to produce lactate with only little amounts of hydrogen (Hung et al., 2011). In the previous chapter this impact by using two bacterial communities derived from manure anaerobic digestate was observed on different lignocellulosic carbohydrates. When simple carbohydrates were used as substrate, the community structure and the associated metabolic patterns at the end of fermentation depended on the inoculum.

For now, only little information is available on the impact of the initial structure of bacterial communities on fermentative metabolic patterns and selection of fermentative bacteria. Moreover, initial mixed culture are always complex and present a high microbial diversity (Maintinguer et al., 2015). Dark fermentation often leads to a simplification of the community by selecting specific bacteria, and more preferentially bacteria that can produce hydrogen. By linking the initial and final bacterial community structures as observed after fermentation, it could be possible to estimate which metabolisms could be performed during the process by only knowing the initial bacterial composition of the inoculum. Such knowledge would help users to choose an inoculum depending on the substrate to favour the targeted fermentative metabolites.

In this context, the present chapter focusses on the impact of various initial bacterial community structures on dark fermentation metabolisms, i.e. the selection process of fermentative bacteria and their related metabolic patterns, in relation with the production of hydrogen and co-products.

For this, five different inocula were used to perform dark fermentation in batch reactors, using simple sugar as carbon source, i.e. glucose. Two inocula were sampled from an activated sludge tank in a wastewater treatment plant. One was used directly after sampling, called *Fresh Aerobic Sludge (FAS)*, whereas the other one was used after two months of storage at 35°C with no feeding, called *Stored Aerobic Sludge (SAS)*. Another inoculum was sampled in the same wastewater treatment plant but in the storage tank of recycled activated sludge, called *Fresh Settled Aerobic Sludge (FSAS)*.

Finally, the two results obtained in the previous chapter from the fermentation of glucose using fresh and stored manure anaerobic digestate, i.e. **FM** and **StM** inocula, were also considered for statistical analysis in this experiment.

For this, batch tests were performed as explained in 2.1.2 after heat shock pretreatment of the inocula. With the aim to limit hydrogen consumption, the process was stopped once the plateau of hydrogen production was reached. Metabolites accumulated in the reactors were analysed at the end of fermentation to characterize the metabolic patterns developed by each inoculum. Bacteria composing each fermentative community were also analysed by high throughput sequencing before and after dark fermentation to see how community structure changed during the process, according to the inoculum used.

The production of hydrogen measured for each condition is first presented and then related to the metabolic patterns observed for the five inocula. Then the selected microbial community structures are confronted to metabolic patterns to observe in which proportion the final bacterial community structure can be related to the metabolism. The relation between the bacterial community structure identified before and after dark fermentation is then discussed in relation to the production of metabolites. Canonical correlation analysis based on the relative abundance and the microbial diversity of bacteria composing each major family generally identified in dark fermentation was proposed and discussed to conclude this comparison.

4.2 Impact of the type of inoculum used on the hydrogen production.

The production of hydrogen was measured every eight hours and was stopped once the maximum of hydrogen production was reached as explained before. These values were obtained at different times according to the type of inoculum, i.e. 2.5 days for *Fresh Aerobic Sludge (FAS)*, 3.7 days for *Stored Aerobic Sludge (SAS)*, 3.5 days for *Fresh Settled Aerobic Sludge (FSASP)*, 4.5 days for *Fresh Manure (FM)* and 3.1 days for *Stored Manure (StM)*. At this time, glucose was converted into metabolites expressed as COD equivalents at $84\pm 3\%$ for FM, $60\pm 5\%$ for StM, and $71\pm 1\%$, $82\pm 4\%$ and $67\pm 3\%$ for FAS, SAS and FASSP, respectively. Gompertz model parameters were estimated according to the dynamics of production of hydrogen presented on Figure 4.2-1 and results are reported on Table 4.2-1.

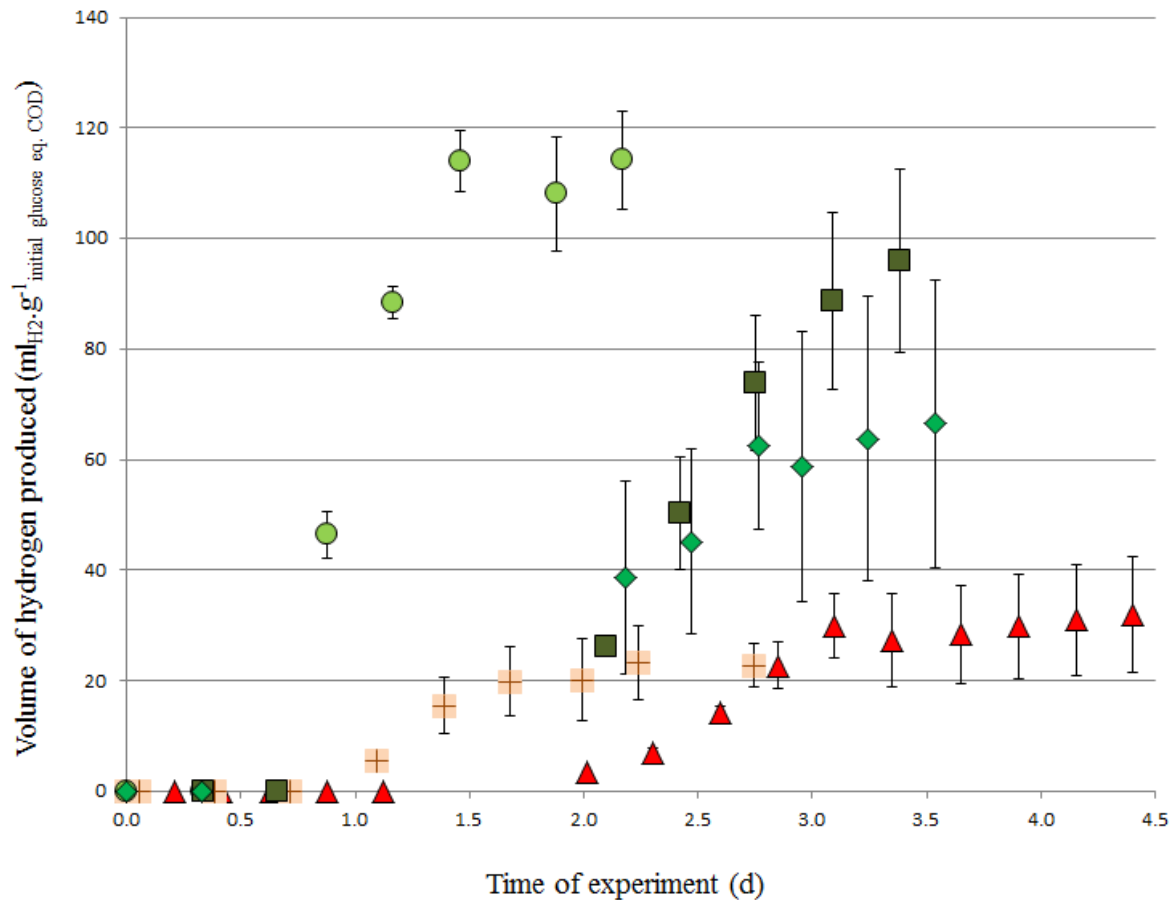


Figure 4.2-1: Amount of hydrogen accumulated during glucose fermentation. Symbols correspond to the type of inoculum used, i.e. FAS (●), SAS (■), FSAS (◆), FM (□), StM (▲).

Interestingly, accumulated amounts of hydrogen differed according to the inoculum used. Maximum amounts of hydrogen accumulated in reactors inoculated with aerobic sludge were 129, 112 and 98 $\text{ml}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{initial COD}}$ for FAS, SAS and FSAS inocula, respectively. These values are more than four times higher than that measured in reactor inoculated manure anaerobic digestate as inoculum. Mixed cultures coming from this activated sludge seems to be more suitable for the production of hydrogen than those coming from manure digestate.

Table 4.2-1: Kinetic parameters of hydrogen production in batch tests according to the initial substrate concentration determined from modified Gompertz equation with P the maximum cumulative hydrogen production ($\text{mlH}_2\cdot\text{g}^{-1}\text{eq initial COD}$), Rm the maximum hydrogen production rate ($\text{mlH}_2\cdot\text{g}^{-1}\text{eq initial COD}\cdot\text{d}^{-1}$), λ the lag phase (days) and R^2 the coefficient of determination of the model. Values correspond to the average of four replicates \pm standard deviation observed between these replicates. Values in % in the H_2 yield column correspond to the proportion of the maximum theoretical value suggested by Hawkes et al. (2007), i.e $2.5 \text{ molH}_2\cdot\text{mol}^{-1}$ degraded substrate in glucose equivalent.

Inoculum	Gompertz equation parameter values				H_2 yield ($\text{mmol}_{\text{H}_2}\cdot\text{g}^{-1}\text{eq. COD consumed}$) and % of the Hawkes maximum theoretical value
	P ($\text{ml}_{\text{H}_2}\cdot\text{g}^{-1}\text{eq. COD initial}$)	Rm ($\text{ml}_{\text{H}_2}\cdot\text{g}^{-1}\text{eq. COD initial}\cdot\text{d}^{-1}$)	λ (days)	R^2	
FM	31 \pm 12	29 \pm 12	1.9 \pm 0.2	0.999 \pm 0.001	1.7 \pm 0.6 (13 \pm 5 %)
StM	23 \pm 2	12 \pm 1	2.1 \pm 0.2	0.999 \pm 0.001	2.2 \pm 0.5 (17 \pm 4 %)
FAS	129 \pm 8	148 \pm 15	0.56 \pm 0.05	0.989 \pm 0.003	7.4 \pm 0.4 (57 \pm 3 %)
SAS	112 \pm 14	79 \pm 1	1.5 \pm 0.3	0.994 \pm 0.004	5.3 \pm 0.6 (41 \pm 5 %)
FSAS	98 \pm 21	104 \pm 38	1.6 \pm 0.3	0.992 \pm 0.003	6 \pm 1 (46 \pm 10 %)

Parameter analysis of hydrogen production dynamics showed that the storage process had an impact on the gas production rate for the activated sludge. The maximum of hydrogen production rate and lag phase evaluated by Gompertz model were indeed higher for the inoculum used directly after sampling than those submitted to different storage, i.e. at lab scale (SAS) and in the WWTP (FSAS). Similar result was previously found for manure digestate inoculum. The production of hydrogen started after half a day of latency for the FAS culture whereas one more day was needed in the case of SAS and FSAS inocula. Such negative impact of inoculum storage on hydrogen production rate was previously shown in a collaborative work performed in the frame of the Biohydrogen Latin America network. As it is well known that bacteria activity after nutrient starvation is lower than unstarved bacteria (Gilbert et al., 1990), the storage might have affected the activity of hydrogen-producing bacteria.

The maximum volume of hydrogen produced per unit of glucose initially introduced was also different when comparing the three aerobic sludge inocula. The highest value was reached by the fresh culture as presented above, and decreased of 13 and 24% for SAS and FSAS, respectively. Hydrogen yields also differed with 7.4, 5.3 and 6 $\text{mmol}_{\text{H}_2}\cdot\text{g}^{-1}\text{consumed COD}$ for FAS, SAS and FSAS respectively.

The bacterial inoculum issued from activated sludge produced higher amount of hydrogen than observed on manure digestate, with some difference when upstream storage was realised. To better understand the mechanisms causing these differences, fermentative metabolic networks were analysed.

4.3 Different metabolic patterns developed by the five inocula during the biological process.

Metabolites that accumulated at the end of fermentation for each inoculum are presented in Figure 4.3-1. Clearly, the metabolic pattern developed by aerobic sludge was different than those observed previously on manure anaerobic digestate. For the three aerobic sludge inocula, butyrate was always the major metabolite produced, representing between 66 and 76% COD equivalent of all metabolites produced. Acetate also accumulated in the reactors and represented about 11% of all products. Butyrate and acetate metabolisms are known as the preferential pathways to produce hydrogen since their production leads to the generation of two and four moles of hydrogen per mole of glucose consumed, respectively (Das, 2001). The major presence of these metabolites in aerobic sludge-based reactor can explain the high amount of hydrogen measured when using these inocula, whereas accumulation of lactate and suspicion of homoacetogenesis resulted in poor production of hydrogen when using mixed culture issued from manure anaerobic digestate (Chapter 3).

a)

Inoculum	Metabolites produced ($\text{mg}_{\text{metabolite eq.COD}\cdot\text{g}^{-1}\text{initial COD}}$)							COD converted (%)
	Acetate	Butyrate	Propionate	Lactate	Formate	Ethanol	Hydrogen	
FM	20±8	116±42	0	592±11	0	44±4	21±8	79±5
StM	213±52	109±10	0	96±36	0	73±26	18±4	51±10
FAS	86±2	532±8	0	0	8.3±0.1	0	84±5	71.2±0.6
SAS	97±25	572±52	63±2	1±1	2±1	59±44	74±9	83±3
FSAS	68±7	493±29	1±1	1±1	4±3	22±20	62±13	66±3

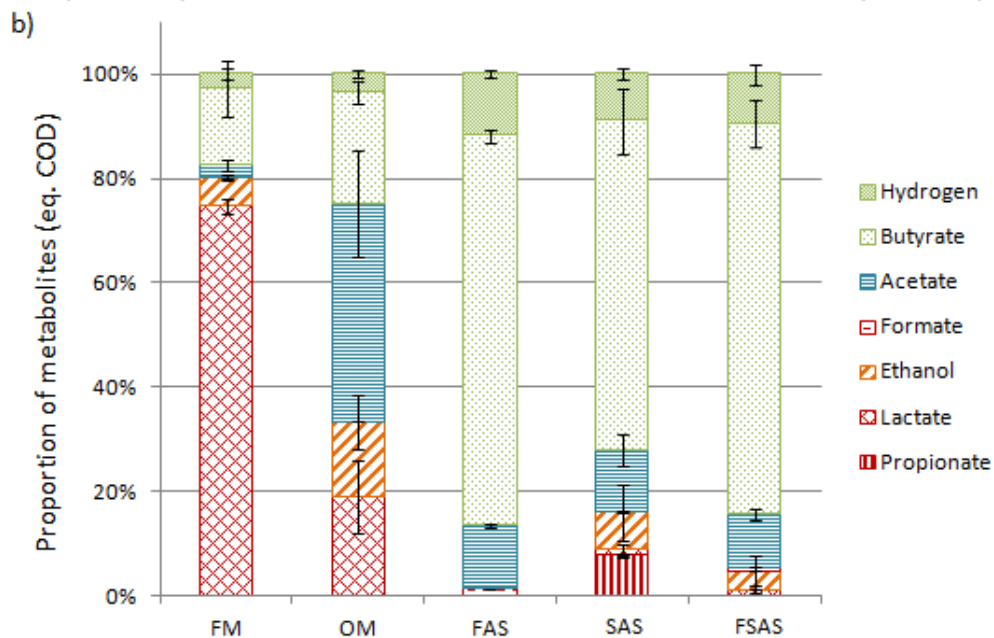


Figure 4.3-1: Metabolites produced during dark fermentation using the five inocula with a) the quantity of each product in $\text{mg}_{\text{metabolite eq.COD}\cdot\text{g}^{-1}\text{initial COD}}$. and b) a graphical representation of their relative proportion for each inoculum. In equivalent COD

Other by products were identified in the reactors inoculated with stored cultures from aerobic sludge, i.e. SAS and FSAS. Ethanol was produced in the two types of reactors, representing 3 and 7% of all metabolites for SAS and FSAS, respectively. As the production of these co-metabolites does not occur with accumulation of hydrogen (Guo et al., 2010), their presence may explain the lower production of H_2 in these reactors than in those inoculated with FAS culture. In the case of SAS-based reactor, 11% of the converted COD corresponded to propionate. The accumulation of this metabolite resulted in lower production of hydrogen in SAS than in FSAS-based reactors. This observation is in

accordance with the consumption of hydrogen during the production of propionate as explained by Latrille *et al.* (2011).

Since different metabolic patterns were observed, reactors were attempted to be grouped according to their fermentative metabolites using a principal component analysis (PCA). The first three principal components explained 86% of the data set variance and results were plotted onto ordination as presented on Figure 4.3-2.

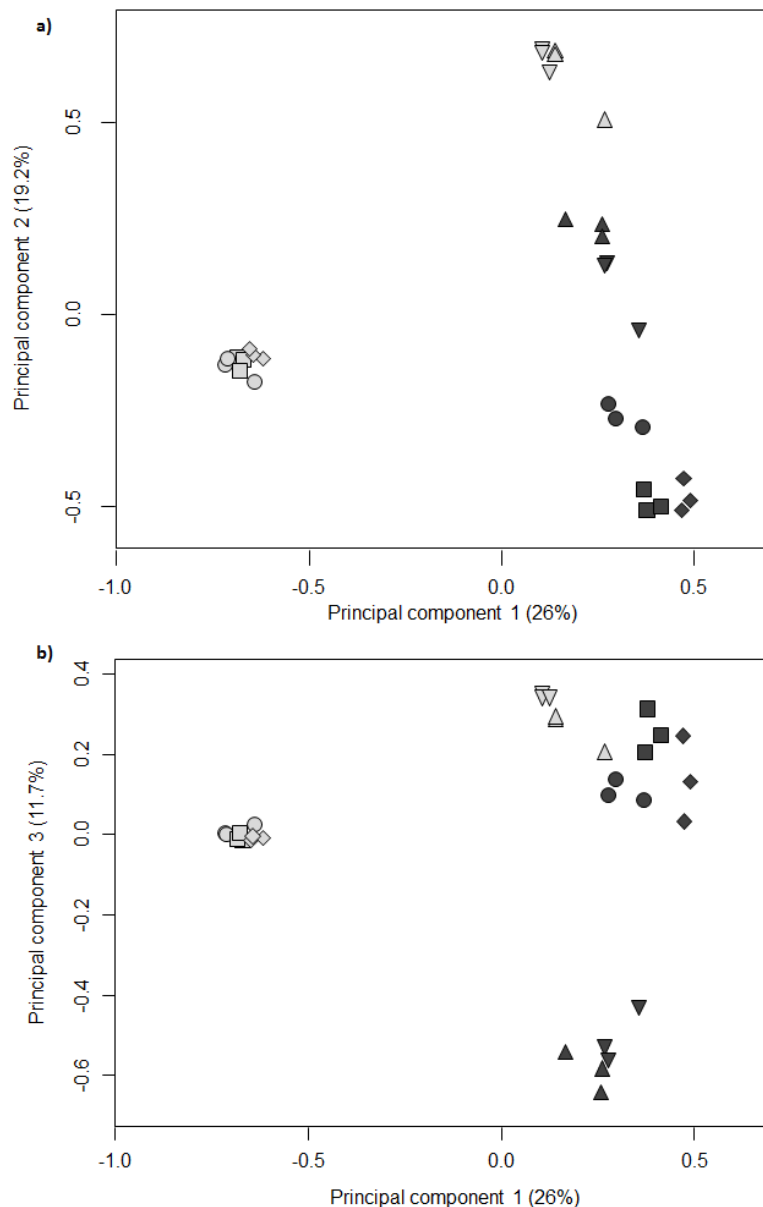


Figure 4.3-2: Principal Components 1, 2 (a) and 1, 3 (b) for the final metabolite composition (in $\text{gCOD produced} \cdot \text{g}^{-1} \text{COD converted}$). In all panels, data corresponding to the five inocula are differentiated by their symbol shape: FM (▲), StM (▼), FAS (●), SAS (■) and FSAS (◆).

Interestingly, samples can be separated according to the two first principal components depending on the main metabolism developed during the process. The three inocula from aerobic sludge are placed on the left side of the ordination, driven by the high amounts of hydrogen and butyrate at the end of the experiment. In contrast, FM samples are located at the right bottom of the figure due to the high proportion of lactate observed in the FM-based reactor. Finally, samples from the StM culture were also located on the right of the ordination in accordance with the accumulation of lactate in these reactors, but on the top since production of acetate and ethanol in these reactors were also higher than in other samples. By adding a third principal component to the analysis (Figure 4.3-1-b), 16.8% more variance can be explained. This component aims to distinguish SAS from FAS samples according to their respective accumulation of propionate and formate.

Overall, each inoculum can be differentiated according to the metabolism they developed during dark fermentation, related to the amounts of hydrogen accumulated in reactor. The metabolism developed by the bacterial consortia is generally the result of a specific bacterial community selected during the process (Akutsu et al., 2009a). Therefore, the bacteria composing fermentative communities selected were analysed and compared to the initial bacterial community structures introduced in reactors to better understand this selection process.

4.4 Structures of bacterial community introduced in reactor and selected fermentative community.

Bacterial compositions of the microbial communities before and after the process of dark fermentation were characterized by amplification and high-throughput amplicon sequencing of partial 16S rRNA. As done in the previous chapter, reactors were attempted to be grouped according their bacterial community structure using PCA based on OTUs identified in samples after Hellinger transformation to reduce the impact of highly minor OTUs on statistical analysis. Results of the multidimensional analysis are presented on the ordination in Figure 4.4-1.

The initial bacterial community structures with the same origin, i.e. aerobic sludge and manure digestate, formed one tight cluster on the first three principal components. The short distance between samples from a same origin suggests that the initial communities introduced in reactors were not that much different, even after storage without feeding. The separation of the three aerobic sludge inocula from the two manure digestate communities shows that the initial community structure were only different according to the inoculum origin.

The distance between communities selected from aerobic sludge inocula and their initial structure confirms that dark fermentation led to a drastic change in bacterial community composition. Distances between samples are much larger after than before fermentation suggesting that the final bacterial communities present significant different structures according to the aerobic sludge inoculum introduced in reactors. In the case of manure digestate-based inocula, structures of bacterial communities were also different before and after dark fermentation since a separation between samples can be observed along the third axe.

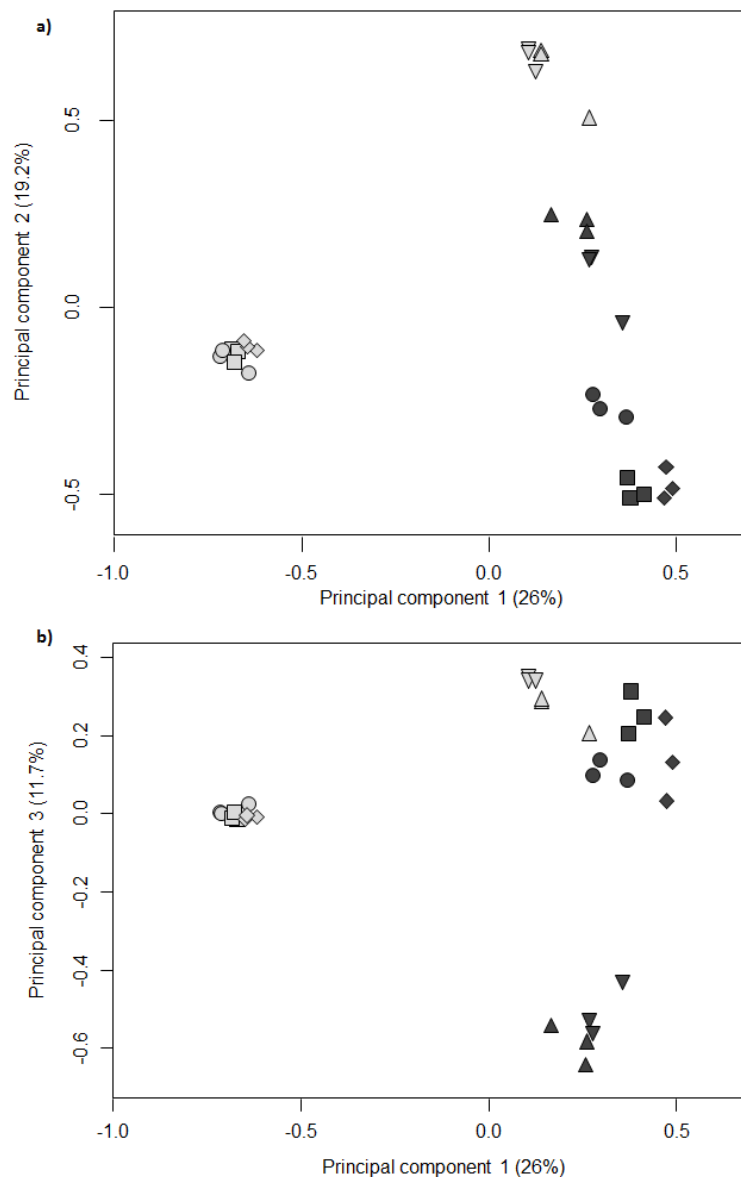


Figure 4.4-1: Principal component analysis (PCA) performed on community structure as absolute abundance of unique sequence types. In all panels, data corresponding to the five inocula are differentiated by their symbol shape: FM (▲), StM (▼), FAS (●), SAS (■) and FSAS (◆). Filled shapes (e.g ●) indicate final compositions after fermentation. Additionally on panels c and d, the inocula structure before fermentation is indicated as light symbols (e.g ○).

The development of the final anaerobic communities went along with a decrease in microbial diversity. The Simpson diversity index was used to quantify this loss. The theoretical range of the Simpson index is between 0 (low diversity) and 1 (high diversity). The initial structures of the five inocula were highly diverse with a Simpson index of 0.98-0.99. At the end of the fermentation, the index decreased for all samples to on average 0.77 ± 0.01 for fresh and 0.90 ± 0.02 for stored manure. Indexes were lower for communities inoculated with aerobic sludge with values at 0.6 ± 0.1 , 0.3 ± 0.2 and 0.6 ± 0.1 for fresh, stored and settled sludge respectively. This decrease of diversity was linked to the emergence of some OTUs as presented on Figure 4.4-2.

Changes of microbial community diversity during fermentation process have already been observed in many studies. During dark fermentation, the culture conditions generally lead to a reduction of diversity by the selection of specific bacteria (Ren et al., 2007). When using simple substrate like glucose, remaining high diversity at the end of the process generally provide hydrogen production fluctuation (Cisneros-Pérez et al., 2015). Substrate competitors or hydrogen-consuming bacteria can be present in these complex communities and lead to poor production of hydrogen, as observed in this study on manure digestate inocula.

To identify the main bacteria composing the bacterial communities selected during fermentation, the relative abundance of the main sequences found at the end of the experiment was presented under the form of a heatmap (Figure 4.4-2).

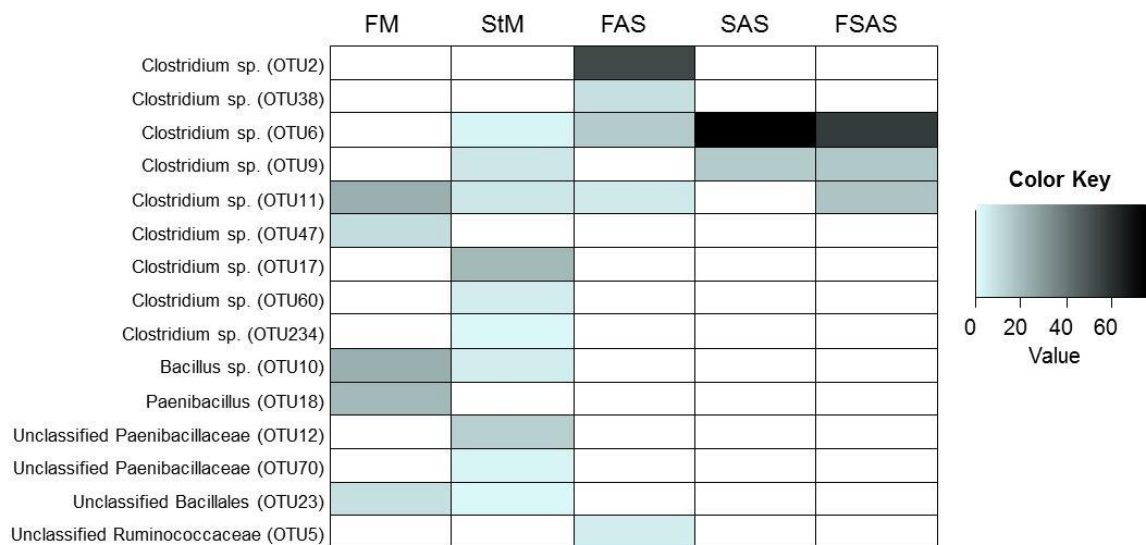


Figure 4.4-2: Heatmap representing the relative abundance of the major sequences (>5%) identified in reactors at the end of the fermentation of glucose with 5 inocula.

This heatmap represents the relative average proportion of the main OTUs (>5% of relative abundance) found at the end of fermentation in all reactors. In the case of the FM culture, about 90% of the relative abundance is shared by five sequences affiliated to both *Clostridium* sp. and *Bacillales* orders. Same observation can be done on StM community with different sequences affiliated to the same taxonomic families. The selection of bacteria affiliated to both *Clostridiales* and *Bacillales* orders is consistent with the low hydrogen amount observed in these reactors. Indeed, the presence of *Paenibacillaceae* and *Bacillaceae* is generally related to lactate or ethanol/acetate metabolisms (Navarro-Díaz et al., 2016). Hydrogen could accumulate through the butyrate-producing pathway due to the presence of *Clostridium* sp. in equivalent relative amount in the medium.

Bacterial selection in aerobic sludge-based culture was more drastic than observed on the two manure inocula. Indeed, between two and four OTUs affiliated to *Clostridium* sp. were selected on FAS, SAS and FSAS, represented 85, 76 and 75% of the identified sequences. Interestingly, one sequence related to *Ruminococcaceae* family represented more than 5% of relative abundance in reactors inoculated with FAS. The identification of this bacteria in reactor fed with simple substrate is surprising since they are generally found in complex biomass as discussed in the previous chapter. It suggests that these hydrolytic bacteria can also emerge on simple substrate even in presence of other competitors.

The identification of bacteria affiliated to *Clostridium* sp. in the three aerobic sludge cultures is consistent with the metabolisms described in the previous section. Indeed, bacteria from this genus are able to convert glucose into butyrate with concomitant accumulation of hydrogen (Rajhi et al., 2013).

Overall, the bacterial community analyses showed that the five inocula used to perform dark fermentation could be grouped into two categories according their origin, i.e. aerobic sludge or manure digestate. The upstream storage performed did not lead to strong differences of initial community structure but the low variability between the slight differences in bacteria composition of these inocula was enough to select different types of communities during dark fermentation that will develop their own metabolism. The next section will focus on the link that can relate the initial bacterial community structure to the one developed after dark fermentation, in accordance to the fermentative metabolic patterns observed.

4.5 Correlation between initial bacterial structure and metabolic performances

The use of various initial bacterial community structures had an impact on metabolic patterns and production of hydrogen during dark fermentation of glucose. Here the aim is to link the community structure of the initial inoculum to the final one and the related global fermentative metabolism.

For this, correlation test between the initial and final community structures and their metabolites at the end of the fermentation was performed using a Mantel test. The test compares the distance matrixes in pairs under the null hypothesis H_0 that 'the matrixes are not correlated'. For the three tests, the Mantel statistics r_M were higher than the level of significance α of 1% (Table 4.5-1) indicating that the correlations between data sets were significant.

Table 4.5-1: Result of Mantel test after 9999 permutations. r_M correspond to Mantel statistic value, α is the risk at 1%.

	Final communities	Final metabolites
Initial communities	$r_M=0.7232$ $\alpha=0.330$ significance of test= 1.10^{-4}	0.8363 0.350 1.10^{-4}
Final communities	ND	0.685 0.324 1.10^{-4}

The bacterial community composing reactors at the beginning and at the end of the fermentation can both explain the metabolic patterns previously observed. But the large diversity of bacteria composing each community inoculated in reactors makes difficult to find the correlation between introduced bacteria and metabolisms observed. Nonetheless, the previous results have shown that bacteria selected after fermentation are mostly affiliated to the six following taxonomic families: *Clostridiaceae*, *Bacillaceae*, *Paenibacillaceae*, *Enterobacteriaceae*, *Ruminococcaceae* and *Lachnospiraceae*. A literature review shows that bacteria from these families are likely functionally important in dark fermentation communities (1.3.4). By grouping sequences based on these family taxonomic affiliation, a simpler data set can be obtained, which might simplify the visualisation of the bacterial community structures and so facilitate the determination of correlations between initial and selected community structures, and fermentative metabolism.

To build this new data set, both OTU relative abundances and diversity Simpson indexes were calculated for each sample from the initial data set of unique OTUs in absolute abundance, once sequences from the same family taxonomic affiliation were grouped together. Relative abundance gave a quantitative value of the presence of the family within the bacterial community. Diversity Simpson index was used to integer the number of main bacteria composing the group. All sequences not affiliated to one of these six families presented above were pooled together in the same group called "others". To compare group of sequences from the same family, ratio of the previous value calculated, i.e. relative abundance and Simpson diversity index, was performed for each sample. A total of 40 "biological index" were calculated as described in Table 4.5-2.

This new data set was calculated for both initial and final bacterial communities found before and after fermentation, respectively. Each matrix was then confronted to the metabolites produced during dark fermentation using a regularised Canonical Correlation Analysis (rCCA). The correlation matrixes resulting from this analysis were represented under the form of a heatmap (Annex I and Annex II). Eleven principal indexes appeared to be highly correlated to the fermentative metabolites produced. For more readability, they were represented alone in Figure 4.5-1 for both initial and final communities.

Table 4.5-2: Description of the biological indexes calculated from the sequences identified by high throughput sequence (Illumina MiSeq). These indexes were both calculated for bacterial initial and final communities

<u>Biological index</u>	<u>Description</u>
<i>Clostridiaceae</i> (rel)	Relative abundance of OTUs from <i>Clostridiaceae</i> family
<i>Bacillaceae</i> (rel)	Relative abundance of OTUs from <i>Bacillaceae</i> family
<i>Lachnospiraceae</i> (rel)	Relative abundance of OTUs from <i>Lachnospiraceae</i> family
<i>Paenibacillaceae</i> (rel)	Relative abundance of OTUs from <i>Paenibacillaceae</i> family
<i>Enterobacteriaceae</i> (rel)	Relative abundance of OTUs from <i>Enterobacteriaceae</i> family
<i>Ruminococcaceae</i> (rel)	Relative abundance of OTUs from <i>Ruminococcaceae</i> family
Other (rel)	Relative abundance of OTUs from other families
<i>Bacillaceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Bacillaceae</i> and <i>Clostridiaceae</i> families
<i>Lachnospiraceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Lachnospiraceae</i> and <i>Clostridiaceae</i> families
<i>Paenibacillaceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Paenibacillaceae</i> and <i>Clostridiaceae</i> families
<i>Enterobacteriaceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Enterobacteriaceae</i> and <i>Clostridiaceae</i> families
<i>Ruminococcaceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Ruminococcaceae</i> and <i>Clostridiaceae</i> families
<i>Clostridiaceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Clostridiaceae</i> and other families
<i>Lachnospiraceae</i> (rel) / <i>Bacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Lachnospiraceae</i> and <i>Bacillaceae</i> families
<i>Paenibacillaceae</i> (rel) / <i>Bacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Paenibacillaceae</i> and <i>Bacillaceae</i> families
<i>Enterobacteriaceae</i> (rel) / <i>Bacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Enterobacteriaceae</i> and <i>Bacillaceae</i> families
<i>Ruminococcaceae</i> (rel) / <i>Bacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Ruminococcaceae</i> and <i>Bacillaceae</i> families
<i>Bacillaceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Bacillaceae</i> and other families
<i>Lachnospiraceae</i> (rel) / <i>Paenibacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Lachnospiraceae</i> and <i>Paenibacillaceae</i> families
<i>Enterobacteriaceae</i> (rel) / <i>Paenibacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Enterobacteriaceae</i> and <i>Paenibacillaceae</i> families
<i>Ruminococcaceae</i> (rel) / <i>Paenibacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Ruminococcaceae</i> and <i>Paenibacillaceae</i> families
<i>Paenibacillaceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Paenibacillaceae</i> and other families
<i>Lachnospiraceae</i> (rel) / <i>Enterobacteriaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Lachnospiraceae</i> and <i>Enterobacteriaceae</i> families
<i>Ruminococcaceae</i> (rel) / <i>Enterobacteriaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Ruminococcaceae</i> and <i>Enterobacteriaceae</i> families
<i>Enterobacteriaceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Enterobacteriaceae</i> and other families
<i>Ruminococcaceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Ruminococcaceae</i> and other families

<u>Biological index</u>	<u>Description</u>
<i>Lachnospiraceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Lachnospiraceae</i> and other families
<i>Clostridiales</i> (rel) / <i>Bacillales</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Clostridiales</i> and <i>Bacillales</i> orders
<i>Clostridiales</i> (rel) / <i>Enterobacteriales</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Clostridiales</i> and <i>Enterobacteriales</i> orders
<i>Bacillales</i> (rel) / <i>Enterobacteriales</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Bacillales</i> and <i>Enterobacteriales</i> orders
Bacteria div.	Simpson diversity index calculated on all identified sequences
Other div.	Simpson diversity index calculated on other sequences not affiliated to the six families selected
<i>Clostridiaceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Clostridiaceae</i> family
<i>Bacillaceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Bacillaceae</i> family
<i>Enterobacteriaceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Enterobacteriaceae</i> family
<i>Lachnospiraceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Lachnospiraceae</i> family
<i>Paenibacillaceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Paenibacillaceae</i> family
<i>Ruminococcaceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Ruminococcaceae</i> family
<i>Bacillaceae</i> div. / <i>Clostridiaceae</i> div.	Ratio of Simpson diversity index calculated from sequences affiliated to <i>Bacillaceae</i> and <i>Clostridiaceae</i> families
<i>Enterobacteriaceae</i> div. / <i>Clostridiaceae</i> div.	Ratio of Simpson diversity index calculated from sequences affiliated to <i>Enterobacteriaceae</i> and <i>Clostridiaceae</i> families
<i>Enterobacteriaceae</i> div. / <i>Bacillaceae</i> div.	Ratio of Simpson diversity index calculated from sequences affiliated to <i>Enterobacteriaceae</i> and <i>Bacillaceae</i> families

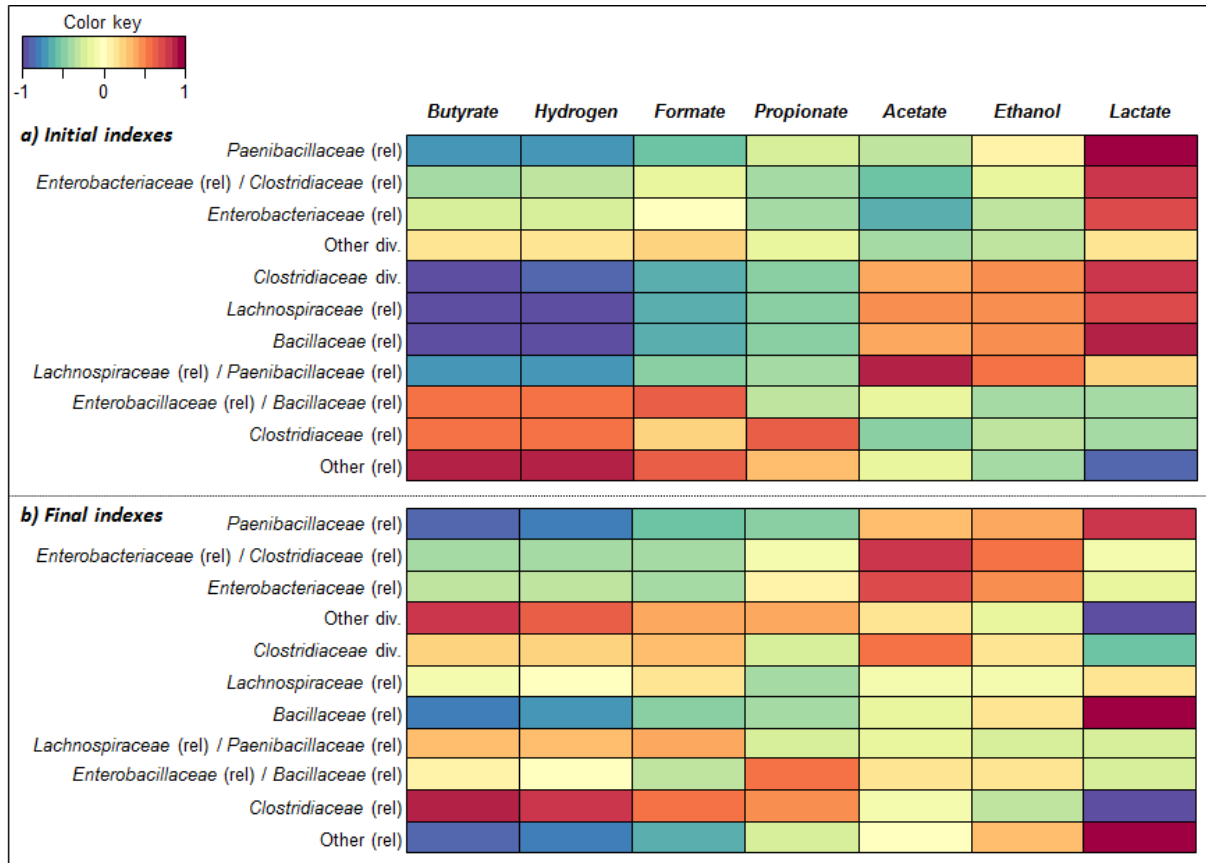


Figure 4.5-1: Major correlations found by rCCA by confronting indexes calculated from a) sequences identified from inoculum and b) sequences identified in selected communities, to metabolites produced during dark fermentation. This figure is only a sample of the total indexes tested. The full heatmaps are presented in Annex I and Annex II.

According to the rCCA performed on initial biological indexes, the production of hydrogen, mainly linked to butyrate pathway, positively correlated with a high relative abundance in bacteria from *Clostridiaceae* and other families (Figure 4.5-1-a). The production of H₂ had also a positive correlation with the ratio of *Enterobacteriaceae* on *Bacillaceae* relative abundance. In contrast, high proportion of bacteria affiliated to *Bacillaceae*, *Paenibacillaceae* and *Enterobacteriaceae* compared to *Clostridiaceae* negatively correlated with H₂ production but were linked to the presence of lactate. Interestingly, negative correlation was also found between a high initial diversity of bacteria from *Clostridiaceae* and hydrogen production. Moreover, a high initial relative abundance of *Lachnospiraceae* bacteria was also related to poor amount of hydrogen and butyrate although this family belongs to the same taxonomic order than *Clostridiaceae*, i.e. *Clostridiales* order.

When looking on the bacterial community structure selected after fermentation (Figure 4.5-1-b), it appears that the production of hydrogen and butyrate also correlated with high relative abundance of *Clostridiaceae* bacteria and negatively correlated with high presence of *Bacillaceae* and

Paenibacillaceae. However, high proportion of bacteria from other families in the final culture was linked to low amounts of hydrogen and butyrate since the correlation turned to negative values, but their high diversity seems to promote the H₂ production.

According to these observations, it can be concluded that the hydrogen-producing bacterial communities selected by dark fermentation must be composed in majority of bacteria affiliated to *Clostridiaceae* family. The large presence of these bacteria in hydrogen-producing mixed culture has been often reported (Arreola-Vargas et al., 2013; Jo et al., 2007). Hydrogen can be produced if *Enterobacteriaceae* are initially present in higher relative abundance than *Bacillaceae* but according to these results, their abundance must be lower than *Clostridiaceae*. The presence of members from other families in the initial community seemed to promote the production of hydrogen. This suggests that positive interactions between these microorganisms might occur in the reactor, contributing to high accumulation of hydrogen. Such effect has already been observed in continuous dark fermentation using mixed culture (Rafrafi et al., 2013). The presence of other bacteria not affiliated to *Bacillales* order can impact the system by directing the metabolism to exclusive production of butyrate, acetate and hydrogen.

At the end of the fermentation, communities having high proportions of *Bacillaceae* and *Paenibacillaceae* are linked to the production of lactate, in accordance to what observed in the literature (Navarro-Díaz et al., 2016). As positive correlation was also found in the initial inoculum between these bacteria and lactate, it suggests that *Bacillaceae* and *Paenibacillaceae* have to be already present in major abundance in the inoculum to produce this metabolite.

By comparing the final to the initial rCCA, a high relative abundance in *Enterobacteriaceae* was not anymore correlated to the production of lactate but to the presence of both ethanol and acetate in the medium. Bacteria from this family were generally found in ethanol-type fermentation with production of hydrogen (Fang, T. Zhang, H. Liu, 2002; Hung et al., 2011). The absence of correlation with these bacteria and H₂ in the present study at the end of the fermentation can be explained by the large proportion of *Clostridiaceae* that are correlated to the production of hydrogen through the butyrate metabolic pathway.

By grouping identified bacteria according to their family taxonomic affiliation, it was possible to visualise more easily the global bacterial community structure of mixed culture. Relevant correlation could be done between biological indicators calculated from the relative abundance of each family in the community and their Simpson diversity index, and the metabolism observed during dark fermentation. A recent study also demonstrated similar relation between bacteria family composing

the community selected after dark fermentation and metabolisms observed (Navarro-Díaz et al., 2016). This work was realised on results obtained from literature review on hydrogen production by mixed culture dark fermentation and confirm correlations that was highlighted in this chapter. In addition, the present study has showed for the first time that similar statistical approach can be done to relate initial community structure to the future metabolism.

4.6 Conclusion

In this chapter, the impact of different inocula on dark fermentation performed on glucose was studied. It was observed that various amounts of hydrogen were obtained depending on the microbial culture used. The differences in hydrogen production were linked to the selection of specific bacterial communities that developed their own metabolism. Consistently with the literature, communities rich in *Clostridium* sp. bacteria led to high accumulation of hydrogen, through a butyrate-producing pathway. When bacteria affiliated to *Paenibacillaceae* and *Bacillaceae* family were present in relative amount similar to *Clostridium* sp., other metabolisms competitive to the production of the hydrogen emerged, i.e. lactate-producing pathway and homoacetogenesis.

The calculation of specific biological indexes based on sequences family affiliation gave an easier visualisation of the structure of mixed culture communities inoculated in reactor and selected after the fermentation process. Regularised canonical correlation analysis (rCCA) performed on these indexes showed that hydrogen production communities were related to the presence of *Clostridiaceae* in higher proportion than *Bacillaceae* or *Paenibacillaceae*, in both initial and final inoculum. At the contrary, when this ratio was reversed, accumulation of lactate was observed. Such correlations have been realised for the first time on initial inoculum community structure. By implemented other results, it could possible to create an efficient tool to predict metabolisms once knowing the proportion of the main bacterial family in the inoculum.

***Chapter 5. Selection of hydrolytic cultures able to
perform hydrogen production by dark
fermentation***

5.1 Context and objective

In previous chapters, it has been shown that hydrolytic bacteria affiliated to *Ruminococcaceae* family were preferentially selected during dark fermentation of complex substrate. Unfortunately, long time required for complex biomass hydrolysis led to low accumulation of hydrogen within an experiment time of at least two weeks. In contrast, simpler substrates, i.e. carbohydrate monomers or dimers led to higher hydrogen production with a specific selection of hydrogen-producing consortia, rich in *Clostridiaceae*.

In this chapter, it was attempted to enrich an inoculum in both hydrolytic and hydrogen producing bacteria that can realise their respective biological activity to degrade complex substrate while producing significant amount of hydrogen.

For this, dark fermentation process was carried out on glucose polymers. Semi-amorphous cellulose (SAC) was chosen as polymeric carbohydrate to enrich the culture in hydrolytic bacteria (SAC reactors). SAC was preferred to microcrystalline cellulose (MCC used in previous experiments) to simplify the system since amorphous structures are more accessible to enzymes than crystalline structures. Cellobiose was selected, as easier biodegradable substrate, to promote the emergence of hydrogen producing community as observed before (CB reactors). This dimer of glucose (and not glucose itself) was selected in order to maintain a hydrolytic activity in all reactors. A mix of these two substrates was finally used to select a mixed community able to both hydrolyse polymer and produce hydrogen (MIX reactors). Experiment was carried out in fed-batch reactors in order to increase the time of experiment and enrich the community in fermentative bacteria without affecting the anaerobic conditions.

Dark fermentation was performed in four replicates with heat-shocked aerobic sludge. This inoculum was used directly after sampling at the WWTP since the highest hydrogen production potential were found with fresh activated sludge as presented in the previous chapter (Chapter 5).

The production of hydrogen in all reactors was monitored every eight hours until a plateau production was reached. In order to minimize hydrogen consumption, head space was purged with nitrogen every day. At the maximum of hydrogen production, 20 ml of medium were sampled for metabolites and bacterial community analysis, and 50 ml of new medium composed of oligo-elements were added in all reactors to avoid any lack of ions. In reactors fed with cellobiose, i.e. CB and MIX reactors, the dimer was also added to a final concentration of $2 \text{ g}_{\text{cod}} \cdot \text{l}^{-1}$. When necessary, pH

was raised to 6 with 2N NaOH. Five cycles of sampling and feeding were performed, representing six hydrogen production cycles, as shown in Figure 5.1-1.

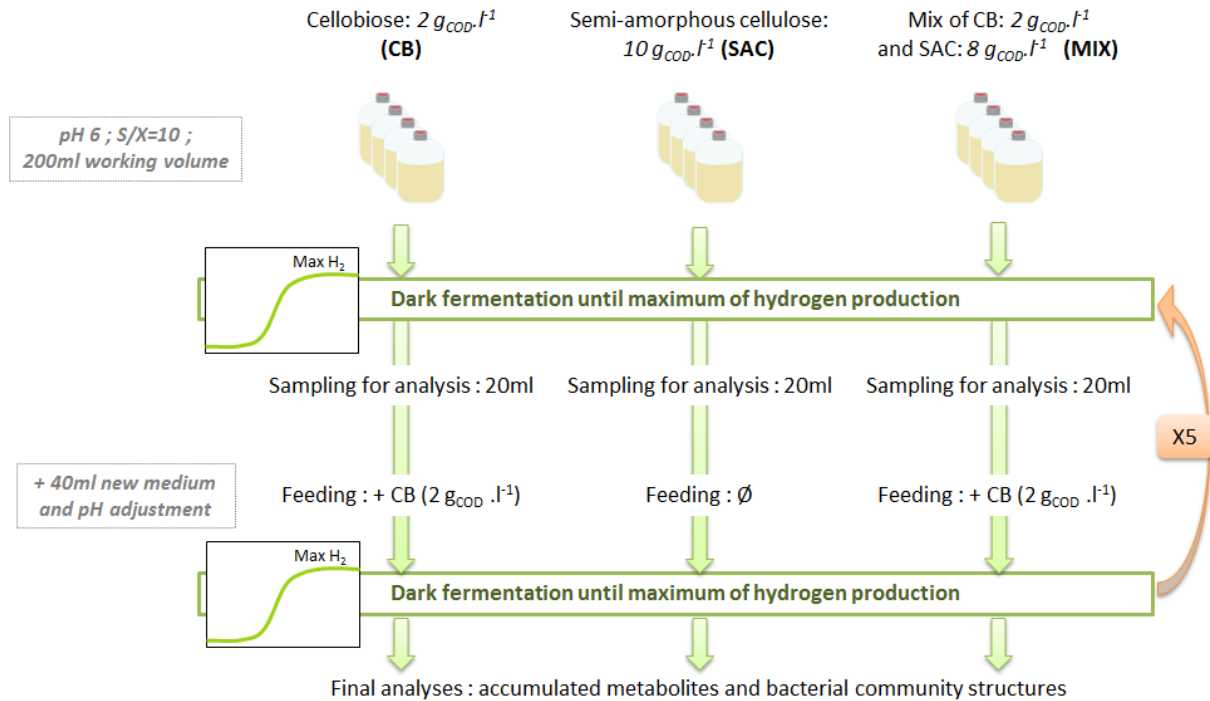


Figure 5.1-1: Fed-batch experimental procedure performed in the present chapter.

First, substrate conversion into fermentative metabolites and hydrogen production were assessed for the three types of reactors along fed-batch cycles. Dynamics of bacterial community structure was then analysed along fed-batch cycles to explain fermentation mechanisms observed during the experiment.

5.2 Analysis of biological activity during the experiment

5.2.1 Increase of substrate conversion yield during fed-batch dark fermentation

The duration of each fed-batch cycle corresponded to 3, 4, 3, 4, 6 and 12 days of fermentation, equivalent to a total of 32 days of experiment from cycle C1 to cycle C6. Metabolites that accumulated in the medium were measured at the end of each cycle and their cumulative quantities in equivalent COD were used to quantify the conversion yield of substrate introduced (Table 5.2-1).

Table 5.2-1: Quantity of COD recovered under the form of metabolites at the end of each cycle (C1 to C6) per gram of initial COD introduced for the three types of reactor and MIX reactor when only considering COD from cellobiose (fourth line). Standard deviation represents the difference between four replicates

Substrate	Number of cycle (total time of experiment in day)					
	C1 (3d)	C2 (7d)	C3 (10d)	C4 (14d)	C5 (20d)	C6 (32d)
Cellobiose $g_{eq\ COD} \cdot g^{-1}_{initial\ CB\ eq.\ COD}$	0.65±0.02	0.70±0.05	0.71±0.03	0.73±0.04	0.81±0.04	0.91±0.02
SAC $g_{eq\ COD} \cdot g^{-1}_{initial\ SAC\ eq.\ COD}$	0.015±0.002	0.24±0.03	0.40±0.04	0.51±0.06	0.58±0.06	0.73±0.05
Mix $g_{eq\ COD} \cdot g^{-1}_{initial\ SAC + CB\ eq.\ COD}$	0.150±0.004	0.24±0.01	0.31±0.01	0.32±0.03	0.37±0.02	0.51±0.02
Mix $g_{eq\ COD} \cdot g^{-1}_{initial\ CB\ eq.\ COD}$	0.76±0.02	0.78±0.03	0.84±0.03	0.79±0.07	0.87±0.04	1.16±0.05

Substrate conversion into metabolites equivalent COD was observed in the three types of reactors, with an increase of conversion yields noticed all along the cycles. Whereas 65% of cellobiose eq. COD introduced for the first cycle was transformed into metabolites, the final conversion of this substrate at the end of the sixth cycles was of 90%. In the case of reactors fed with semi-amorphous cellulose (SAC), significant amount of metabolites was measured at the end of the second cycle. Interestingly, SAC was converted during all fed-batch cycles to reach a final value of 73% of conversion yield. Similar conversion yield on cellulose was already observed using inoculum adapted to lignocellulosic residues degradation such as rumen microbial consortium (Hu et al., 2004). The present study showed that aerobic sludge inoculum can also reach high polymer conversion yield during long term dark fermentation, i.e. 32 days in this case.

When SAC was co-fermented with cellobiose, final substrate conversion yield was lower than noticed for the two previous types of reactor. Only 51% of substrates introduced were transformed into metabolites after 32 days of fermentation. As cellobiose has a lower degree of polymerisation than SAC, it might preferentially be degraded by the anaerobic flora. Substrate conversion yield of mix reactors was also calculated considering only the COD brought by cellobiose to compare with results obtained when the dimer was introduced as sole carbon source. Values obtained are also higher than those observed in only cellobiose-based reactors. This parameter exceeded the maximum of $1\ g_{COD\ produced} \cdot g^{-1}_{introduced\ COD}$ at the end of the last cycle, suggesting that some SAC was also degraded during the fermentation but at a lower extent than alone. Indeed, by difference, SAC conversion in mix reactor appeared to be lower than when fermented alone. The presence of an easier degradable sugar seems to negatively affect the polymer conversion. Previous study observed

that expression of hydrolytic enzymes request to release sugar from polymer were repressed when bacteria grew on simple carbohydrate (Macfarlane et al., 1990). In the case of cellulose, cellulase required to hydrolyse the polymer are inhibited in presence of cellobiose (Philippidis et al., 1993). However, the inhibitory effect of cellobiose is eliminated once cellobiose is converted into glucose (Brethauer and Studer, 2015). In the present study, increase of SAC conversion in MIX reactors was observed at the end of the two last fed-batch cycles, when longer time of fermentation led to the total degradation of cellobiose and so allowed the conversion of SAC (Table 5.2-1).

5.2.2 Accumulation of hydrogen along the fed-batch cycles and associated metabolic patterns

More particularly, the cumulative amount of hydrogen obtained along the experiment was analysed for the three types of reactors (Figure 5.2-1). To compare the results, the quantity of hydrogen was expressed in ml of hydrogen per gram of COD introduced in the medium. In the case of reactors fed with the mixture of substrates, a fourth curve was drawn based the production of hydrogen when only considering the added cellobiose COD; units were here expressed in ml of hydrogen produced per gram of cellobiose equivalent COD introduced. Metabolic patterns were concomitantly analysed at the end of each cycle (Figure 5.2-2).

In reactors fed with cellobiose, the production of hydrogen started during the first cycle with a maximum of $135 \pm 9 \text{ ml}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{COD}}$ measured after two days of fermentation. The production was associated to an acetate/ethanol metabolism (Figure 5.2-2), known to lead to the accumulation of hydrogen (REN et al., 2006). After a second feeding, the production was lower since only $33 \pm 16 \text{ ml}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{COD}}$ accumulated in the headspace, although the metabolism turned to butyrate production, a hydrogen producing metabolic pathway. No production of hydrogen was detected during the third cycle, but the production resumed during the fourth cycle. However, amount of hydrogen produced remained lower than observed at the end of the first cycle since a maximum of $12 \text{ ml}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{initial COD}}$ was obtained. The production remained constant on cycles 5 and 6 with 44 and 36 $\text{ml}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{initial COD}}$, respectively.

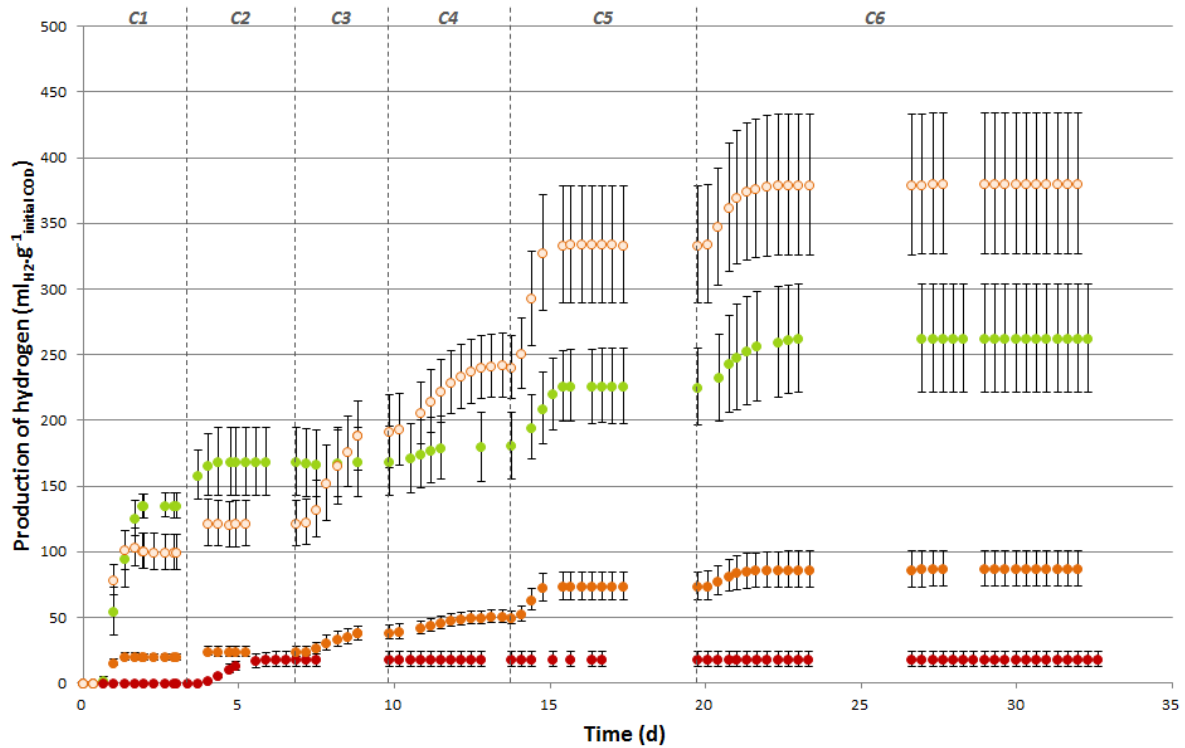
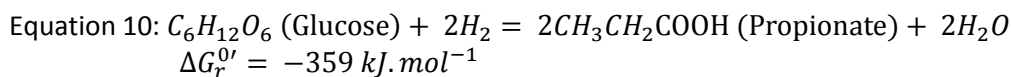


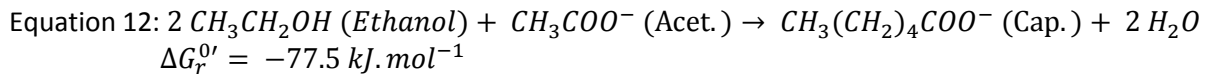
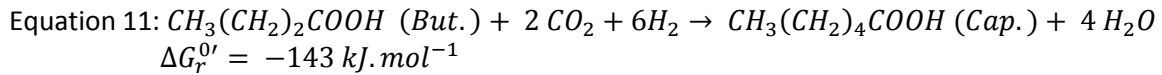
Figure 5.2-1: Cumulative amount of hydrogen obtained along the different fed-batch cycles for SAC (●); Cellobiose (●); Mix (●) and Mix if considering only the COD from cellobiose (○), in $\text{ml}_{\text{H}_2} \cdot \text{g}^{-1}_{\text{initial COD}}$. Error bars correspond to the standard deviation observed between four replicate. Dotted lines represent a new cycle start, i.e. the insertion cellobiose in the medium.

The decrease of hydrogen production could be explained by a metabolic shift since propionate and caproate were also found in the medium. Propionate metabolic pathway is associated to the consumption of H_2 as described in Equation 10 (Latrille et al., 2011).



At the end of the experiment, $91 \text{ mg}_{\text{propionate eq. COD}} \cdot \text{g}^{-1}_{\text{COD introduced}}$ accumulated in the medium, equivalent to 0.81mmol of propionate. According to the previous stoichiometric equation, the volume of hydrogen needed for the production of propionate might be equivalent to 18ml of hydrogen.

The production of caproate during dark fermentation has already been observed in previous studies (Quéméneur et al., 2011b) but the metabolic pathway remained unclear. Latrille *et al.* (2011) suggested that caproate production occurs from butyrate and hydrogen consumption (Equation 11), whereas production pathway through ethanol and acetate consumption has been observed from *Clostridium kluyveri* according to Equation 12 (Ding et al., 2010).



With butyrate as *But.*, caproate as *Cap.*, and acetate as *Acet.*

In the case of the present study, ethanol concentration decreased along cycles and was not anymore identified at the end of the last cycle. For this reason, the production of caproate by consumption of ethanol seems to occur during this experiment. However, if the production of caproate occurred with the consumption of hydrogen, the $44 \text{ mg}_{\text{caproate eq. COD}} \cdot \text{g}^{-1}_{\text{COD introduced}}$ produced during the fermentation would prevent the accumulation of about 23 ml of hydrogen. By considering hydrogen consumption through propionate and caproate metabolic pathways, only 41 ml more of hydrogen could be obtained at the end of 32 days experiment, representing a total of 304 ml of H_2 per gram of COD introduced. This value is quite lower than what it could be obtained if the production of hydrogen observed during the first fed-batch cycle remained constant all over the different cycles, which represents a maximum of 810 ml of hydrogen. Consequently, other hydrogen consuming metabolic pathways may be present but not identifiable such as homoacetogenesis (Saady, 2013).

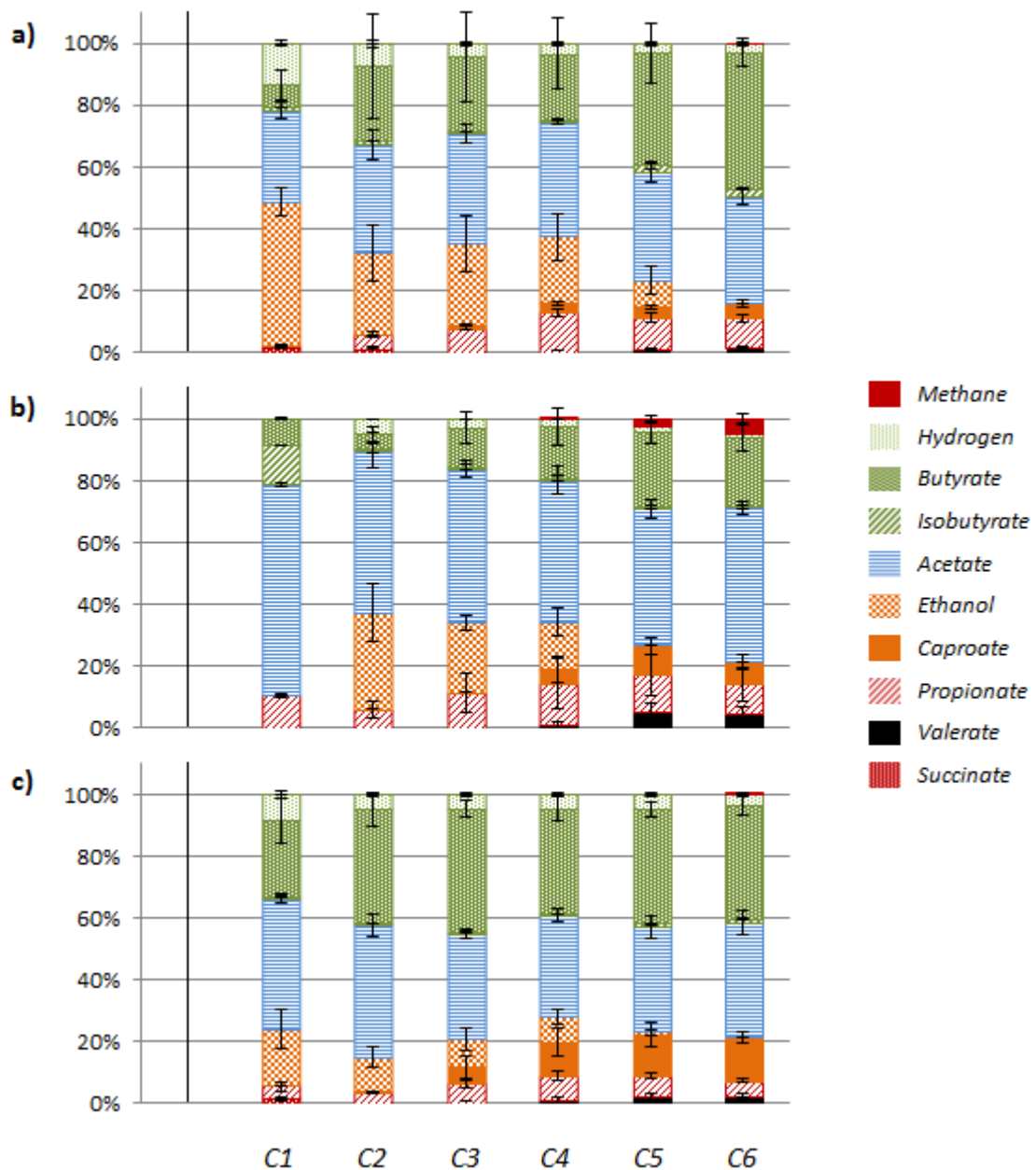
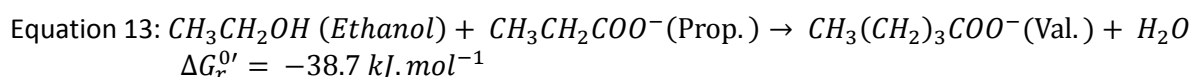


Figure 5.2-2: Relative proportion of metabolites identified at the end of each fed-batch cycle (C1 to C6) in equivalent COD for reactors fed with cellobiose (a), SAC (b) and mix of these two substrates (c). Error bars correspond to the difference measured between replicates.

As observed before, low amounts of hydrogen were produced during the conversion of SAC. No hydrogen and only few metabolites were detected during the first cycle, in relation with low substrate conversion yield of 1.5% (Table 5.2-1). The production of the hydrogen started during the second cycle with the same metabolic pattern as observed for cellobiose-based reactors, i.e. hydrogen production through acetate/ethanol pathway. No more hydrogen was measured until the end of the experiment even though butyrate accumulated in the medium. The apparition of metabolites whose production required the consumption of hydrogen, i.e. propionate and methane,

could explain the absence of H₂. Considering the amount of propionate and methane observed at the end of the sixth cycle 54 ml more of hydrogen could be obtained at the end of the experiment per gram of COD added, or a total of 72ml of hydrogen at the end of the experiment. However, the production of methane is surprising since the mixed culture was heat-shock pre-treated before inoculation in order to inactivate methanogens activity. It seems that long term dark fermentation, at least 14 days, is enough to re-activate methanogenic archaea. This phenomenon has already been observed by Luo *et al.* (2011) who reported a production of methane during repeated batch dark fermentation after the same time of experimentation.

As observed in previous reactors, caproate production appeared in the same time as ethanol consumption, i.e. from the fourth cycle, suggesting a metabolic shift during the experiment. Accumulation of valerate was also observed at the end of cycle 5 and 6. As for caproate, the production of valerate can be obtained by the consumption of ethanol and propionate as shown in Equation 13 (Ding *et al.*, 2010), two metabolites firstly produced in the present study.



Hydrogen production in reactors containing both cellobiose and SAC was observed all along the experiment (Figure 5.2-1). To compare with cellobiose-based reactor, hydrogen productions were also calculated considering only the dimer of glucose. During the first cycle of experiment, a maximum of 20 ml_{H₂}·g⁻¹_{Total COD} accumulated in the headspace, equivalent to 99 ml_{H₂}·g⁻¹_{COD} if only cellobiose is considered. A similar metabolism as developed in cellobiose-based reactors was observed. As before, less hydrogen accumulated during the second cycle, but the production increased after the third feeding. It was associated to higher amounts of butyrate when compared to the cellobiose-based reactors. Indeed, 0.33 g_{butyrate eq. COD}·g⁻¹_{initial COD} were produced if considering only COD from cellobiose in MIX reactors whereas 0.17g_{butyrate eq. COD}·g⁻¹_{initial COD} were obtained in the case CB reactors.

As observed on previous substrates, accumulation of propionate, caproate and valerate was also observed during the fed-batch cycles, suggesting that some H₂ was lost during the fermentation. Considering the hydrogen potentially consumed for the production of caproate and propionate, 20 ml more of hydrogen could be obtained per gram of COD added or a total of 400ml of hydrogen at the end of the sixth fed-batch cycle.

As observed in reactors fed with only SAC, methanogenesis appeared at the end of the last cycle, 18 days after its detection in SAC reactors. The presence of easier biodegradable substrate with the polymer might slow down the emergence of a methanogenic activity. The negative impact of soluble sugars on methanogens has been observed in anaerobic digestion since their transformation into volatile fatty acids promotes acidification of the medium and subsequent methanogens inhibition (Motte et al., 2015).

By co-fermenting a polymeric carbohydrate, i.e. SAC, and a simpler substrate, cellobiose, it was possible to maintain the production of hydrogen all along the experiment while degrading the polymer. Hydrogen was produced in comparable amount as obtained on the simple substrate alone, and even exceed this value at the end of the experiment. To visualise the bacterial communities selected during the fed-batch dark fermentation, biomolecular analysis were performed at the end of each fed-batch cycles.

5.3 Bacterial communities selected in each reactor along the experiment.

5.3.1 Structure of the initial bacterial community used as inoculum

As observed in the previous section, hydrogen producing metabolism observed during the first fed-batch cycle was different than those discussed in Chapter 5. The production of hydrogen occurred here through the production of acetate and ethanol whereas hydrogen was observed during the production of butyrate in Chapter 5. Such difference can be due to the use of different inoculum bacterial community structures at the beginning of the experiment. To explore this hypothesis, initial bacterial community structure of inoculum used in this study was confronted to inocula used in the previous chapter by principal component analysis (PCA). Result was plotted into the ordination in Figure 5.3-1.

Interestingly, the three aerobic sludge inocula used in the previous chapter are pooled together and are separated from manure digestate inocula, with and without storage, along the first component. The aerobic sludge inoculum used in the present study formed a distinct group along the two first components. This observation confirms that the inoculum used in this study had a different bacterial community structure than observed for FAS inoculum, although it was sampled in the same aerobic tank of the WWTP and used directly after sampling, i.e. without any storage. The only difference between the two inocula was the sampling season since the first inoculum was sampled in January and the second in April of the same year.

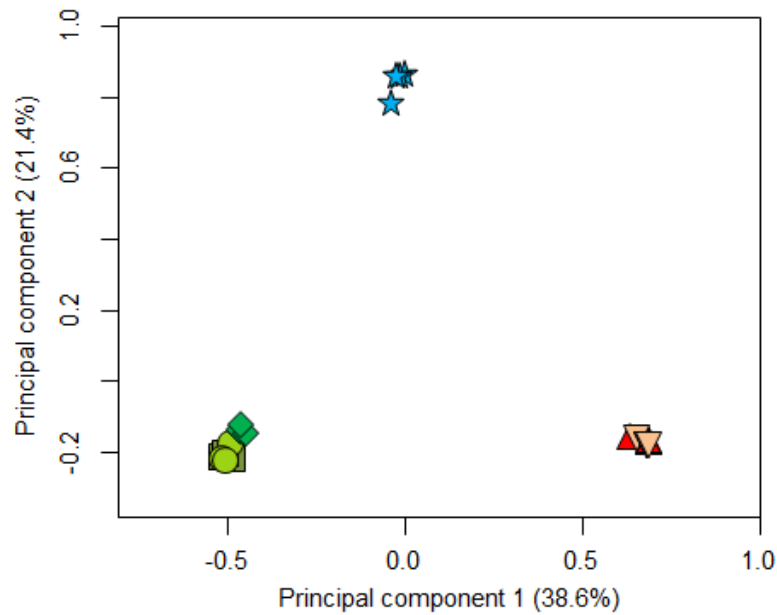


Figure 5.3-1: PCA performed on initial bacterial community structure inoculated in reactors. Green and orange symbols represent samples used in Chapter 5, i.e. FAS (●), SAS (■), FSAS (◆), FM (▲), StM (▼). Blue stars correspond to the inoculum community structure used in the present chapter (★).

Deeper analysis of the bacterial community showed that mainly two sequences affiliated to *Enterobacteriaceae* family were identified in the FAS-Ap inoculum sampled in April, presenting 50% of relative abundance, whereas bacteria from *Clostridiaceae* family mainly composed the FAS inoculum sampled in January. Seasonal changes in microbial community structure have already been reported in many studies dealing with activated sludge. A recent study performed in Madison (WI, USA), country located at the same latitude as Narbonne (France), showed that bacterial community composing activated sludge in WWTP depended mainly on the season, i.e. winter, spring, summer and autumn (Flowers et al., 2013). Such difference of initial community structure might affect the dark fermentation mechanisms.

However, no deeper comparison could be done between the two initial community structures, FAS and FAS-Ap, and the metabolisms observed after the first batch cycle as done in Chapter 4 by calculation of biological indicators, because the fermentation was carried out on two different substrates. Contrary to glucose, cellobiose degradation requires a first hydrolysis step before conversion into fermentation metabolites that could affect the selection of bacteria and the final metabolism. Because of this, biological indicators designed in the previous chapter were not calculated on initial bacterial community structure in the present study.

5.3.2 Bacterial community change along fed-batch cycles

As bacteria family affiliation was presented as an efficient taxonomic level to explain the bacterial community structure, OTUs identified in each reactor were grouped according their family affiliation. The relative abundance of family groups was represented under the form of heatmap in Figure 5.3-2.

As explained before, the inoculum used was composed of 50% of *Enterobacteriaceae* whereas the rest of the relative abundance was shared between bacteria from other families. *Clostridiaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Bacillaceae* and *Paenibacillaceae* were also found in the inoculum but their cumulative relative abundance was below 5% and thus cases were uncoloured on the heatmap.

During the experiment, *Clostridiaceae* bacteria were identified at the end of all cycles with an average relative abundance of 16, 24, and 31% for reactors fed with SAC, cellobiose and mix of substrates, respectively. Proportion of sequences identified as *Enterobacteriaceae*, *Ruminococcaceae* and *Lachnospiraceae* evolved during fed-batch cycles with different dynamics for the three types of reactor.

Bacteria from *Enterobacteriaceae* family were identified as major family at the end of the first cycles for the two types of reactor containing cellobiose, with 59 and 62% of relative abundance for cellobiose and mix substrate-based reactors, respectively. In contrast, their abundance was below 5 % in SAC-based reactors. From the second feeding and all along fed-batch cycles, their relative abundance decreased in favour of *Ruminococcaceae* in cellobiose and mix reactors. However, in the case of MIX reactors, the proportion of *Clostridiaceae*, *Enterobacteriaceae* and *Ruminococcaceae* is quite equivalent from the third to the fifth cycle, about 30% of relative abundance, whereas *Ruminococcaceae* represented 61% of the total community for CB reactor at the end of the fourth cycle.

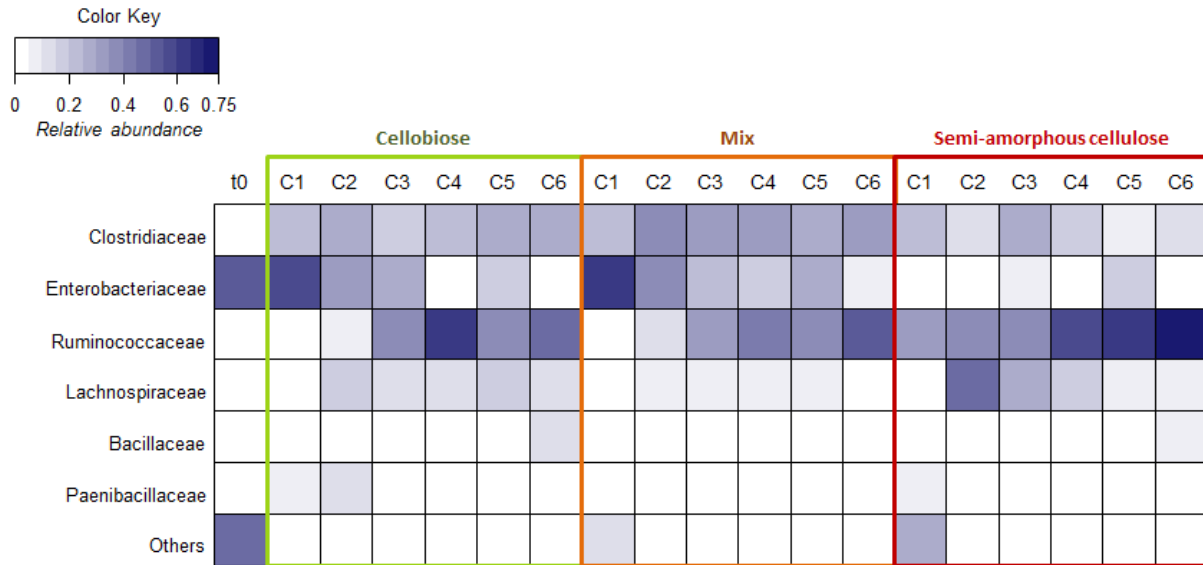


Figure 5.3-2: Graphical representation of relative abundance of main fermentative bacteria families identified in dark fermentation at the end of each fed-batch cycle (C1 to C6) for the three types of reactors. White colour corresponds to relative abundance below to 5%.

In the case of community developed on SAC, bacteria affiliated to *Clostridiaceae* and *Ruminococcaceae* families were the major bacteria identified from the first cycle. At the end of the second cycle, *Lachnospiraceae* were found in major relative abundance. During the third cycle, shift from *Lachnospiraceae* to *Ruminococcaceae* bacteria was observed. Emergence of *Ruminococcaceae* bacteria during the experiment is a common feature of the three types of reactors.

In the present study, bacterial community structure changes were identified all along the experiment, related to metabolic shifts due to the consumption of some metabolites during the successive fed-batch cycles. Because of this, the realisation of PCA as done in the previous chapters did not provide significant results and could not be used. Consequently, another statistical approach than PCA was preferred to visualise temporal dynamics of the bacterial communities during fed-batch cycles. The Nonmetric Multidimensional Scaling (NMDS) is a statistical method which represents similarities among objects based on the rank order of variables composing each sample. Because variable weight is not considered by this approach, NMDS has been widely used to identify gradients such as temporal gradients (Ramette, 2007). NMDS was performed on all the OTUs identified after sequencing and Bray-Curtis distance was used to represent object dissimilarities, with a suitable stress value of 0.19. Distances were projected onto two-dimensional plot presented in Figure 5.3-3 for both samples (a) and sequences (b) data.

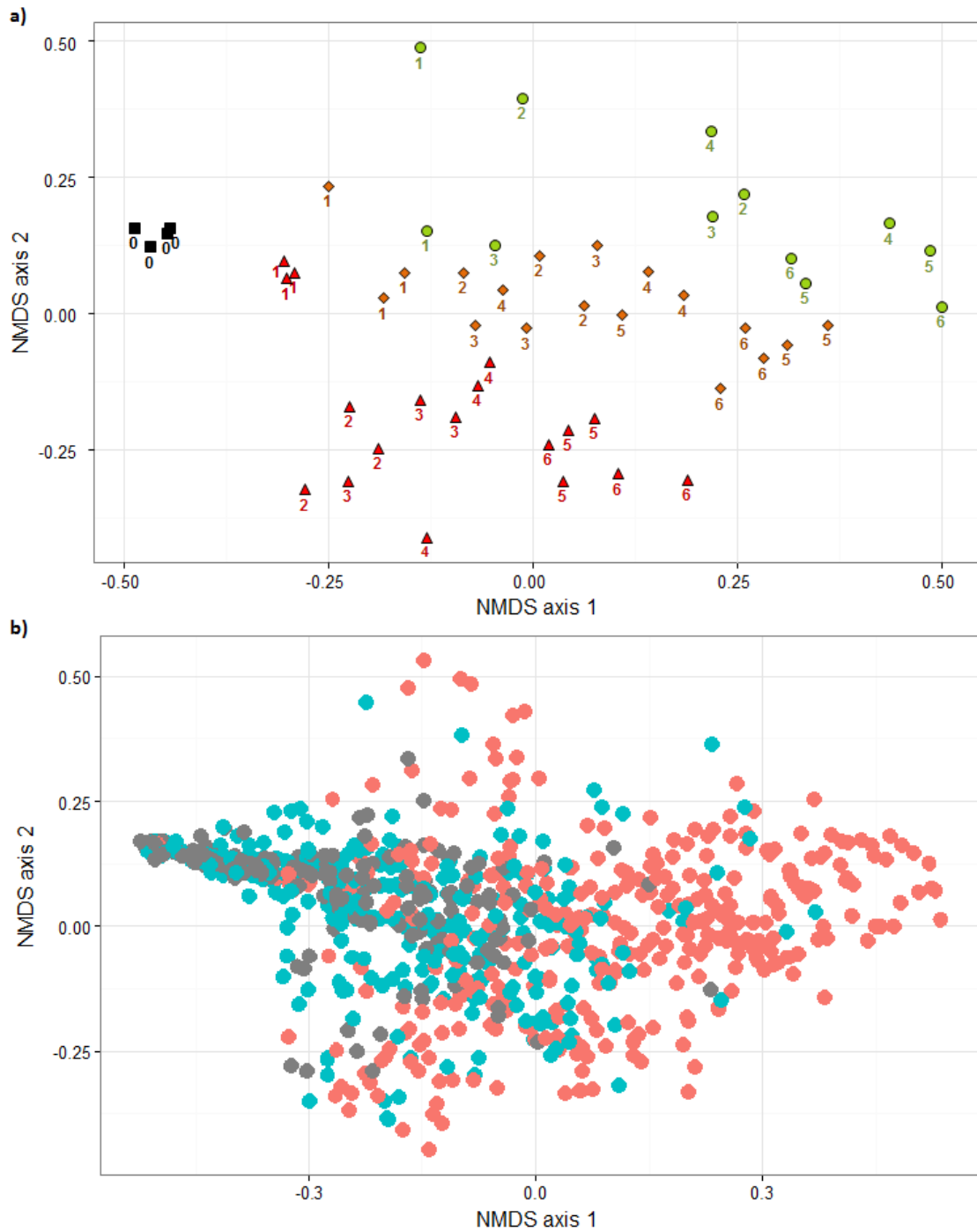


Figure 5.3-3: Ordination graph for a two-dimensional NMDS of the identified sequences based on the Bray-Curtis distance. The first plot (a) represents similarities between samples along the different cycles (number below symbols) for reactors fed with cellobiose (●), with SAC (▲), and with a mix of these two substrates (◆). The second graph (b) represents similarities between identified sequences affiliated to *Firmicutes* (●), *Proteobacteria* (●) and other taxonomic phyla (●).

On representation of sample distance (Figure 5.3-3-a), it can be observed that objects are placed along the first NMDS axis according to temporal gradient. Initial communities are placed on

the left side of the ordination and move on the right side along the fed-batch cycles. This change of community structure is supported by a shift from sequenced affiliated to Proteobacteria, to Firmicutes phyla (Figure 5.3-3-b). The transition from Proteobacteria to Firmicutes bacteria seems to appear around the second and third fed-batch cycle, which is in accordance to the metabolic shift observed in the previous section.

On the second axis, reactors seem to be separated according to the substrate fermented (Figure 5.3-3-a). Indeed, reactors fed with SAC are placed in the negative values whereas communities developed on cellobiose are in the positive values. Interestingly, reactors fed with the two substrates are placed between the two other types of reactors, suggesting that communities developed on SAC and cellobiose shared similarities with the two communities identified on pure substrates. However, along the experiment, distance between the different reactors decreased, suggesting a convergence of bacterial community to a similar structure. According to the previous observation, this convergence is due to the emergence of bacteria from *Ruminococcaceae* family, belonging to *Firmicutes* phylum.

This result suggests that, even considering sugar with low degree of polymerisation, the use of fed batch that increased fermentation time of experiment led to an inevitable emergence of *Ruminococcaceae*. However, by co-fermenting carbohydrate polymer and a simpler sugar, it was possible to maintain a co-culture of *Enterobacteriaceae* and *Ruminococcaceae* during 32 days of fermentation that permitted to produce significant amount of hydrogen while hydrolysing the biomass.

5.3.3 Link between metabolic patterns and selected community structures after fed-batch cycles

As selected communities at each fed-batch cycle resulted of different culture conditions, i.e. fermentation of different substrates, correlations between selected community and fermentation metabolism could be explored using regularised canonical correlation analysis (rCCA), as done in the previous chapter. To better explore the bacterial community structure, the biological indicators designed in the previous chapter were calculated for each bacterial community identified after each cycle. The rCCA was used to investigate correlation between each cycle metabolic pattern and the calculated biological indicators. For more clarity, results of rCCA were presented for the first cycle where the hydrogen producing metabolism was observed, the third cycle to represent the metabolic shift, and the sixth cycle to show the final metabolism. As some metabolites were identified for the

three cycles and other metabolites were identified in only of some of them, metabolites were divided into two groups, i.e. metabolites common and specific of each cycle. The main correlations obtained are presented under the form of heatmap on Figure 5.3-4 and description of indicators in .

Table 5.3-1: Description of the main biological indexes calculated from the sequences identified by high throughout sequence (Illumina MiSeq).

<u>Biological index</u>	<u>Description</u>
<i>Clostridiaceae</i> (rel)	Relative abundance of OTUs from <i>Clostridiaceae</i> family
<i>Lachnospiraceae</i> (rel)	Relative abundance of OTUs from <i>Lachnospiraceae</i> family
<i>Enterobacteriaceae</i> (rel)	Relative abundance of OTUs from <i>Enterobacteriaceae</i> family
<i>Ruminococcaceae</i> (rel)	Relative abundance of OTUs from <i>Ruminococcaceae</i> family
<i>Clostridiaceae</i> (rel) / <i>Bacillaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Bacillaceae</i> and <i>Clostridiaceae</i> families
<i>Lachnospiraceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Lachnospiraceae</i> and <i>Clostridiaceae</i> families
<i>Enterobacteriaceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Enterobacteriaceae</i> and <i>Clostridiaceae</i> families
<i>Ruminococcaceae</i> (rel) / <i>Clostridiaceae</i> (rel)	Ratio of relative abundance of OTUs affiliated to <i>Ruminococcaceae</i> and <i>Clostridiaceae</i> families
<i>Clostridiaceae</i> (rel) / Other (rel)	Ratio of relative abundance of OTUs affiliated to <i>Clostridiaceae</i> and other families
Bacteria div.	Simpson diversity index calculated on all identified sequences
<i>Ruminococcaceae</i> div.	Simpson diversity index calculated on sequences affiliated to <i>Ruminococcaceae</i> family
<i>Enterobacteriaceae</i> div. / <i>Clostridiaceae</i> div.	Ratio of Simpson diversity index calculated from sequences affiliated to <i>Enterobacteriaceae</i> and <i>Clostridiaceae</i> families
<i>Enterobacteriaceae</i> div. / <i>Bacillaceae</i> div.	Ratio of Simpson diversity index calculated from sequences affiliated to <i>Enterobacteriaceae</i> and <i>Bacillaceae</i> families

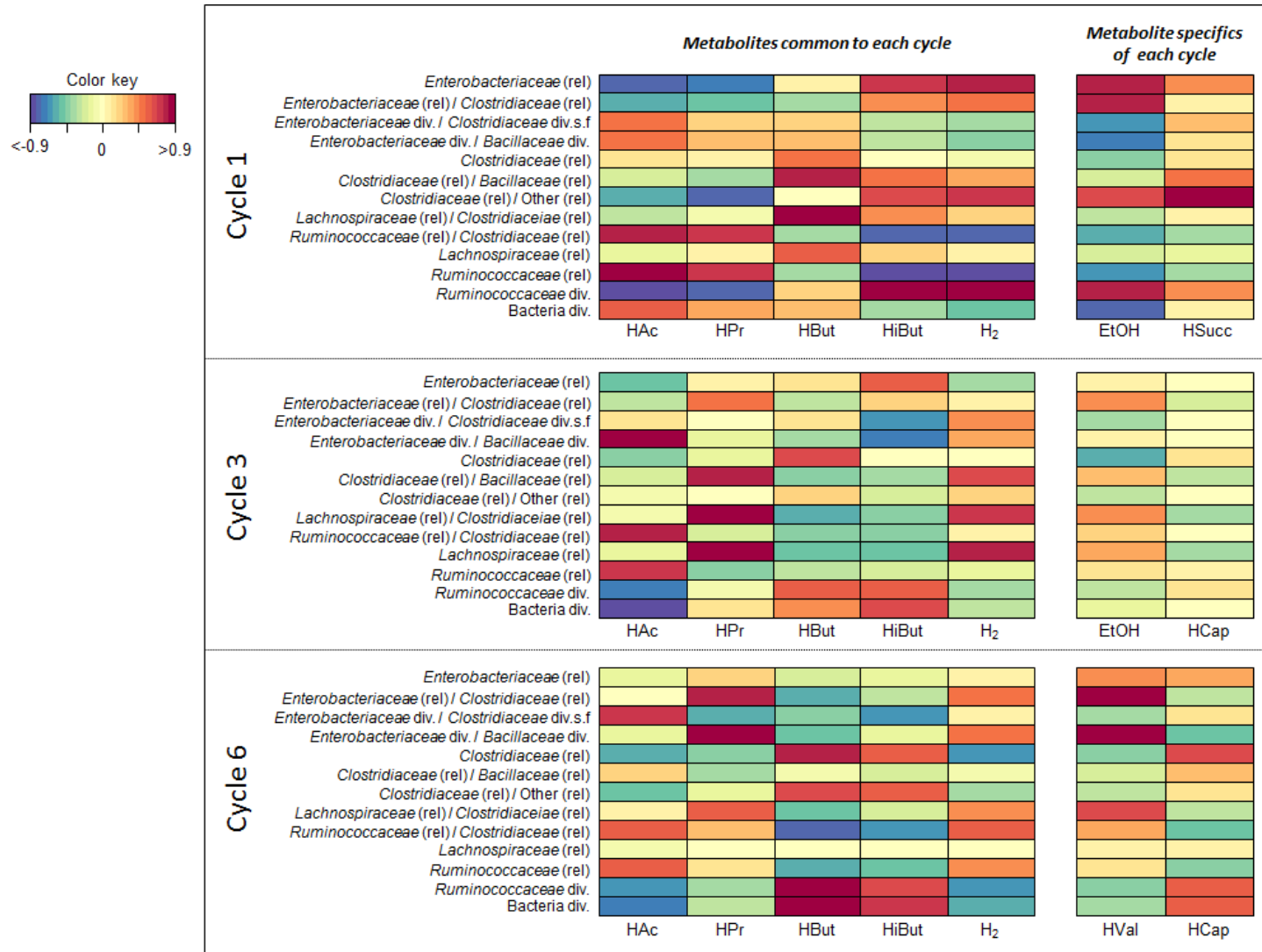


Figure 5.3-4: Regularised Canonical Correlation Analysis (rCCA) performed on calculated indicators

During the first cycle, a positive correlation can be observed between the production of hydrogen and high relative abundance of *Enterobacteriaceae*. High relative abundance of *Clostridiaceae* is also related to the production of hydrogen but when present in higher relative amount than bacteria from other families. These indicators are also positively correlated to the production of ethanol. A positive correlation between *Enterobacteriaceae* and the production of ethanol was also observed in the previous chapter when glucose was used as substrate. But in the case of glucose, no link with hydrogen production could be done because of the large presence of *Clostridiaceae* that were associated to the production of hydrogen through butyrate pathway. Because these two types of bacteria are generally selected together, the role of *Enterobacteriaceae* in the production of hydrogen is unclear (Hung et al., 2011). In the present study, concomitant growth of bacteria from these two families was also observed, but because *Enterobacteriaceae* were present in major proportion than *Clostridiaceae*, correlation with the production of hydrogen was observed. However, this analysis does not permit to determine if *Enterobacteriaceae* are direct producer of hydrogen or if they play a secondary role in the production of this gas, by consuming traces of oxygen or by directly interacting with clostridia species as it was previously observed (Rafrafi, 2012).

Along cycles, the decrease of hydrogen production and consumption of ethanol in CB and MIX reactors conduced to uncorrelated ethanol and hydrogen to these indicators. In the case of the third cycle, hydrogen production is correlated the presence of *Lachnospiraceae*, family identified in SAC reactors when hydrogen was produced. Indeed, bacteria from this family are known to produce hydrogen (Angenent et al., 2004). In addition, H₂ production is positively correlated to a higher proportion of *Clostridiaceae* than *Bacillaceae*. This last correlation was also observed in the previous chapter, which confirms that higher amount of *Clostridiaceae* than *Bacillaceae* are required to produce H₂.

At the contrary, high relative abundance of bacteria from *Ruminococcaceae* family is first negatively correlated to the production of hydrogen and ethanol. Indeed, in the first fed-batch cycle, these bacteria were only identified on SAC when no hydrogen was produced. This correlation can be explained by the initial hydrolysis step required before the production of hydrogen, activity developed by these bacteria. Interestingly, high diversity of *Ruminococcaceae* is positively correlated to the production of hydrogen, suggesting that, at the beginning of fermentation, high number of different *Ruminococcaceae* can promote the hydrogen production as long as their relative abundance is low. All along the fed-batch cycles, high proportion of *Ruminococcaceae* remained

positively correlated to the production of acetate, which is in accordance to the metabolism of *Ruminococcaceae* bacteria (Iannotti et al., 1973).

Surprisingly, butyrate and hydrogen production does not appear to be linked, as it was observed in previous chapters and as it was expected regarding the metabolism. At the contrary, hydrogen seems to be linked to the production of propionate as observed at the end of cycles 3 and 6. Indeed, at these cycles, these metabolites are correlated to the same biological indicators as presented above.

At the end of the last fed-batch cycle, positive correlation appeared between hydrogen production and high proportion of *Ruminococcaceae*, in accordance with the specific selection of these bacteria in all reactors after the sixth cycle and the production of hydrogen in CB and MIX reactors. Interestingly, hydrogen production is also correlated to the presence of *Enterobacteriaceae* in higher relative amount than *Clostridiaceae* and with a higher diversity than bacteria from *Bacillaceae* family. This result confirms that the co-culture of *Enterobacteriaceae* and *Ruminococcaceae* promotes the production of hydrogen.

5.4 Conclusion

In this chapter, the possibility to produce hydrogen by long term dark fermentation in fed-batch process using a glucose polymer as substrate was investigated. When mixed culture was fed with cellobiose, accumulation of hydrogen was observed during all the experiment. Fluctuation of hydrogen production was observed due to a metabolic shift that went along with a change of bacterial community structure. The bacterial community was first composed of bacteria affiliated to *Enterobacteriaceae* family that was associated to the production of hydrogen through acetate-ethanol metabolic pathway. Along the experiment, *Ruminococcaceae* bacteria continuously emerged to represent the most abundant taxonomic family from the third fed-batch cycle. When SAC was used as substrate, *Ruminococcaceae* were present in majority from the first fed-batch cycle and remained the most abundant family all along the fermentation. In this case, the bacterial community selected efficiently converted the SAC as 73% of the substrate eq. COD were transformed into fermentative metabolites at the end of fermentation. But the apparition of a methanogenic activity after 14 days of fermentation resulted in no more production of hydrogen.

When both cellobiose and SAC were fermented by the mixed culture, both hydrolytic and hydrogen producing activities were identified during the 32 days of experiment. In this case, it resulted in maintenance of the co-culture of *Enterobacteriaceae* and *Ruminococcaceae* bacteria

during the time of experiment. Even if the lower degradation of SAC was observed during the co-fermentation of cellobiose and SAC, hydrolytic activity was maintained. However, potential inhibition of hydrolytic enzymes activity by the presence of cellobiose was suggested.

These results suggest that the co-fermentation of complex and simpler sugar can select a bacterial community able to hydrolysis and hydrogen producing activities. In addition, the use of the biological indicators previously created permitted to efficiently link fermentative metabolisms with the bacterial community selected.

General conclusion and outlooks

Hydrogen production from organic waste such as lignocellulosic residues has recently gained interest since it can potentially contribute to a simultaneous production of clean and renewable energy together with sustainable waste management. Based on the natural activity of anaerobic microorganisms, dark fermentation is one of the technics that exist to convert lignocellulose into hydrogen. The aim of the present thesis was to increase knowledge about dark fermentation mechanisms, linking the bacterial community structures with fermentative metabolisms regarding the substrate composition.

By performing batch dark fermentation tests on various **simple carbohydrates** composing lignocellulose, different fermentative metabolisms were observed depending on the type of substrate. As main result, these various metabolisms were due to a selection of specific bacterial community structures during fermentation. For the specific case of a manure digestate-based inoculum, lactate-producing bacteria affiliated to *Paenibacillaceae* and *Bacillaceae* families were mainly identified in reactors fed with hexose-based carbohydrates whereas hydrogen-producing microorganisms from *Clostridiaceae* family were found in higher proportion in reactors fed with pentose carbohydrates. Consequently, more hydrogen was produced from the conversion of pentoses than hexoses. This observation highlighted that the type of soluble carbohydrate in the dark fermentation reactors has an important impact on the process through the selection of specific bacteria having their own metabolism.

Nonetheless, dark fermentation metabolisms are closely related to the type of inoculum used. This observation has been shown in various studies that noticed changes of metabolic pattern according to the initial inoculum, associated to the selection of specific communities (Akutsu et al., 2009a). In the case of the present study, this effect was observed when using the same manure digestate inoculum but after exposure to environmental stress caused by two months of storage. In this case, the stress generated by the starvation period promoted the selection of homoacetogens on all carbohydrates, reducing the amount of hydrogen accumulated compared to the fresh inoculum. When other types of inoculum were used to ferment glucose, different fermentation metabolisms were identified. Hence, by using bacterial consortia from aerobic sludge sampled in wastewater treatment plant (WWTP), an ideal hydrogen-producing metabolism based on butyrate and acetate production was observed, related to the selection of bacteria from *Clostridiaceae* family. This type of metabolism led to the production of 4 to 5 times more hydrogen than observed on the two manure digestate inocula. Storages of activated sludge inoculum at lab-scale and at the WWTP resulted in little variations of the bacterial community structure but led to the development of hydrogen non-producing or consuming metabolic pathways, i.e. propionate and ethanol.

However, characteristics of inoculum bacterial community that affect the bacterial selection, and thus the fermentation metabolism, are still unclear. Because of their complex structure, bacterial communities composing inocula are rarely investigated although they could explain the metabolic pattern and selected community observed after dark fermentation.

In the present study and focussing on glucose dark fermentation, indicators based on sequence family affiliations were proposed for the first time. With a simplified visualisation of initial bacterial community structure, they allowed to link inoculum community structures to fermentative metabolisms that were obtained after dark fermentation. By using these indicators, it was possible to link high production of hydrogen with initial bacterial community rich in *Clostridiaceae* whereas lactate was preferentially produced when bacteria from *Paenibacillaceae* and *Bacillaceae* were present in major relative abundance in the inoculum.

These indicators permitted to effectively explain metabolisms observed on glucose fermentation. As two distinct structures of bacteria were used to test the relevance of these indicators, implementation of additional dark fermentation results obtained from other types of inoculum could substantiate the correlations observed here and highlight other relations between bacterial community and fermentation metabolism.

In the case **polymeric carbohydrates** i.e. microcrystalline cellulose, xylan and wheat straw fermented by the two manure digestates, bacteria affiliated to other taxonomic families than observed on simple carbohydrate were selected during dark fermentation. They were affiliated to *Ruminococcaceae* and, to lesser extent, *Lachnospiraceae* families, and all of them are associated to hydrolytic activity. The low hydrogen production obtained in these reactors was attributed to the presence of *Clostridiaceae* that were identified in lower amount than *Ruminococcaceae*. These results suggested that to produce reasonable amount of hydrogen from complex biomass, bacterial consortia must be composed of both hydrolytic and hydrogen producing bacteria. However, long time fermentation required for polymer hydrolysis is not consistent with production of hydrogen since hydrogen-consuming bacteria appeared once H₂ has accumulated.

To increase the time of dark fermentation and thus promoting the emergence of hydrolytic bacteria, long term dark fermentation was realised using fed-batch process on glucose polymers, i.e. cellobiose and semi-amorphous cellulose (SAC). It was observed that hydrogen producing bacteria were rapidly selected when cellobiose was introduced as sole carbon source in reactors. As observed on manure digestate, hydrolytic bacteria from *Ruminococcaceae* and *Lachnospiraceae* families were selected from the first three days of fermentation when SAC was fermented alone. However, at the

end of the experiment, *Ruminococcaceae* was the most abundant family in all reactors, including those fed with cellobiose. This result suggests that long term dark fermentation on oligo- or polymeric sugars conduces to the convergence of communities to a similar structure, in order to efficiently hydrolyse the carbohydrate, independently the degree of polymerisation of the carbohydrate used. The selection of *Ruminococcaceae* on complex substrates has been observed in two different types of inoculum, i.e. manure digestate and activated sludge, suggesting that the structure of fermentation bacterial community is driven by complex biomass to select an efficient hydrolytic consortium.

By co-fermenting cellobiose with SAC, *Ruminococcaceae* were also identified as main bacterial family at the end of the experiment. However, hydrogen production was maintained during all the experiment and, when considering only the COD coming from cellobiose, in a higher amount than observed in reactors fed with only cellobiose. The production of hydrogen went along with the maintenance of *Enterobacteriaceae* that were associated to the production of hydrogen through acetate-ethanol pathway. Degradation of SAC was also observed but in lower proportion than when the SAC was fermented alone. Consequently, bacterial hydrolytic activity was maintained during the co-fermentation of the two substrates, but some hydrolysis inhibitions due to the presence of cellobiose was suspected.

During this work, the study of the tri-interaction between substrate composition, fermentation metabolisms and bacterial community structure during dark fermentation brought new knowledge on dark fermentation mechanisms. It was shown that the bacterial community structure was driven by the type of substrate. Community structure was more flexible when simple carbohydrate was used as substrate, resulting in different bacteria selection depending on the initial inoculum. In the case of polymeric sugars and complex biomass as wheat straw, bacterial community was driven to the selection of hydrolytic bacteria, regardless the inoculum used. Regarding these results, some research perspective can be suggested.

Dark fermentation performed on mixed culture exposed to storage without feeding showed that this upstream process conduced to low changes of inoculum bacterial community structure but had a significant impact on the selection of fermentative bacteria, associated to different metabolic patterns. To overcome stress and community structure changes when waiting period is required before dark fermentation, upstream inoculum conditioning technics that do not affect the process have to be found. Inoculum freezing or drying are technics proposed in the literature but remain not optimal.

The variation in bacterial community structures during the dark fermentation process due to different types of soluble sugars can constitute a barrier for stable continuous production of hydrogen in upscaled systems. Because of seasonal culture, the composition of agricultural residues in carbohydrate varies all over the year, and can thus provide fluctuation of hydrogen production during dark fermentation. However, the specific selection of bacteria depending on the type of carbohydrate could be used to manage bacterial consortia. Depending on the fermentative bacterial community present in reactors, the introduction of a specific sugar could drive the community to another structure that will develop a different metabolism. That was observed during the co-fermentation of cellobiose and cellulose where hydrogen-producing metabolism was promoted by regular addition of cellobiose. However, the fed-batch process used to perform the selection has to be improved to avoid metabolites accumulation in the liquid phase that provokes metabolism and bacterial community shift during long term dark fermentation. Continuous dark fermentation using immobilised-mixed cultures or membrane reactor could be preferred since, in this system, bacteria are retained in the reactor and effluent containing fermentative by-products is continuously evacuated.

Other bacterial community management strategy by addition of pure exogenous bacterial strains has also demonstrated some successful results (Kotay and Das, 2010). But the use of pure culture is expensive and long term maintenance of these exogenous bacteria in a natural consortium remains unclear. Addition of soluble sugar coming from waste in reactors instead of pure bacterial culture could be an original and cheaper way to control fermentative metabolism and so improve the production of hydrogen by dark fermentation. In the case of heterogeneous polymers such as lignocellulosic biomass, fruit and vegetable waste can represent the source of soluble carbohydrates to avoid the use of pure carbohydrates. These residues constitutes a significant waste deposit since about 20% of harvested fruit and vegetables are wasted every year in the world (FAO, 2011).

To manage bacterial communities through a fine control of available soluble sugars, more information has to be collected about the structure of bacterial community regarding the inoculum and the carbohydrates used. Whereas up to now, studies generally only focused on the bacterial community structures selected after dark fermentation, this thesis showed that it is possible to link fermentation metabolisms with the initial bacterial families composing inocula. To bring more knowledge about bacteria selection during dark fermentation, the present work suggests to analyse the inoculum community structure at family level since this taxonomic rank has been shown as effective to explain the bacterial structure while simplifying the data.

To sum up, this thesis permitted to acquire important information to better understand the interactions between substrate, bacterial community and metabolism. All these results may be applied in the near future, may help to manage bacterial communities and to better control their metabolisms on different substrates.

References

- Adessi, A., De Philippis, R., 2014. Photobioreactor design and illumination systems for H₂ production with anoxygenic photosynthetic bacteria: A review. *Int. J. Hydrogen Energy* 39, 3127–3141. doi:10.1016/j.ijhydene.2013.12.084
- AFHYPAC, 2016a. L'union Européenne et l'hydrogène.
- AFHYPAC, 2016b. Production et consommation d'hydrogène aujourd'hui.
- Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment: Fundamentals toward application. *Biotechnol. Adv.* 29, 675–685. doi:10.1016/j.biotechadv.2011.05.005
- Akutsu, Y., Lee, D.-Y., Li, Y.-Y., Noike, T., 2009a. Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. *Int. J. Hydrogen Energy* 34, 5365–5372. doi:10.1016/j.ijhydene.2009.04.052
- Akutsu, Y., Li, Y.-Y., Harada, H., Yu, H.-Q., 2009b. Effects of temperature and substrate concentration on biological hydrogen production from starch. *Int. J. Hydrogen Energy* 34, 2558–2566. doi:10.1016/j.ijhydene.2009.01.048
- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.* 101, 4851–4861. doi:10.1016/j.biortech.2009.11.093
- Ananyev, G.M., Skizim, N.J., Dismukes, G.C., 2012. Enhancing biological hydrogen production from cyanobacteria by removal of excreted products. *J. Biotechnol.* 162, 97–104. doi:10.1016/j.jbiotec.2012.03.026
- Andersson, J., Lundgren, J., 2014. Techno-economic analysis of ammonia production via integrated biomass gasification. *Appl. Energy* 130, 484–490. doi:10.1016/j.apenergy.2014.02.029
- Androga, D.D., Ozgur, E., Gunduz, U., Yucel, M., Eroglu, I., 2011. Factors affecting the longterm stability of biomass and hydrogen productivity in outdoor photofermentation. *Int. J. Hydrogen Energy* 36, 11369–11378. doi:10.1016/j.ijhydene.2010.12.054
- APHA, 1999. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.
- Arreola-Vargas, J., Celis, L.B., Buitrón, G., Razo-Flores, E., Alatríste-Mondragón, F., 2013.

- Hydrogen production from acid and enzymatic oat straw hydrolysates in an anaerobic sequencing batch reactor: Performance and microbial population analysis. *Int. J. Hydrogen Energy* 38, 13884–13894. doi:10.1016/j.ijhydene.2013.08.065
- Azwar, M.Y., Hussain, M.A., Abdul-Wahab, A.K., 2014. Development of biohydrogen production by photobiological, fermentation and electrochemical processes: A review. *Renew. Sustain. Energy Rev.* 31, 158–173. doi:10.1016/j.rser.2013.11.022
- Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G., 2008. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. *Int. J. Hydrogen Energy* 33, 4064–4073. doi:10.1016/j.ijhydene.2008.05.069
- Bakonyi, P., Nemestóthy, N., Simon, V., Bélafi-Bakó, K., 2014. Review on the start-up experiences of continuous fermentative hydrogen producing bioreactors. *Renew. Sustain. Energy Rev.* 40, 806–813. doi:10.1016/j.rser.2014.08.014
- Ball, M., Weeda, M., 2015. The hydrogen economy - Vision or reality? *Int. J. Hydrogen Energy* 40, 7903–7919. doi:10.1016/j.ijhydene.2015.04.032
- Barakat, A., de Vries, H., Rouau, X., 2013. Dry fractionation process as an important step in current and future lignocellulose biorefineries: a review. *Bioresour. Technol.* 134, 362–73. doi:10.1016/j.biortech.2013.01.169
- Basak, N., Jana, A.K., Das, D., Saikia, D., 2014. Photofermentative molecular biohydrogen production by purple-non-sulfur (PNS) bacteria in various modes: The present progress and future perspective. *Int. J. Hydrogen Energy* 39, 6853–6871. doi:10.1016/j.ijhydene.2014.02.093
- Bastidas-Oyanedel, J.-R.R., 2011. Thermodynamic based modelling of biohydrogen production by anaerobic fermentation Ph.D.
- Behera, S., Arora, R., Nandhagopal, N., Kumar, S., 2014. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renew. Sustain. Energy Rev.* 36, 91–106. doi:10.1016/j.rser.2014.04.047
- Belay, A., Ota, Y., Miyakawa, K., Shimamatsu, H., 1993. Current knowledge on potential health benefits of Spirulina. *J. Appl. Phycol.* 5, 235–241. doi:10.1007/BF00004024
- Benner, R., Maccubbin, A.E., Hodson, R.E., 1984. Anaerobic Biodegradation of the Lignin and

Polysaccharide Components of Lignocellulose and Synthetic Lignin by Sediment Microflora. *Appl. Environ. Microbiol.* 47, 998–1004. doi:0099-2240/84/050998-07\$02.00/0

Benomar, S., Ranava, D., Cárdenas, M.L., Trably, E., Rafrafi, Y., Ducret, A., Hamelin, J., Lojou, E., Steyer, J., Giudici-Orticoni, M.-T., 2015. Nutritional stress induces exchange of cell material and energetic coupling between bacterial species. *Nat. Commun.* 6, 6283. doi:10.1038/ncomms7283

Bettiga, M., Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2009. Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. *Microb. Cell Fact.* 8, 40. doi:10.1186/1475-2859-8-40

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K.M., Sani, R.K., 2013. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresour. Technol.* 128, 751–759. doi:10.1016/j.biortech.2012.10.145

Bhutto, A.W., Bazmi, A.A., Zahedi, G., 2013. Underground coal gasification: From fundamentals to applications. *Prog. Energy Combust. Sci.* 39, 189–214. doi:10.1016/j.pecs.2012.09.004

Brethauer, S., Studer, M.H., 2015. Biochemical Conversion Processes of Lignocellulosic Biomass to Fuels and Chemicals – A Review. *Chim. Int. J. Chem.* 69, 572–581. doi:10.2533/chimia.2015.572

Bridgwater, A.V., 1995. The technical and economic feasibility of biomass gasification for power generation. *Fuel* 74, 631–653. doi:10.1016/0016-2361(95)00001-L

Brown, D., Rowe, A., Wild, P., 2014. Techno-economic comparisons of hydrogen and synthetic fuel production using forest residue feedstock. *Int. J. Hydrogen Energy* 39, 12551–12562. doi:10.1016/j.ijhydene.2014.06.100

Bugg, T.D.H., Ahmad, M., Hardiman, E.M., Rahmanpour, R., 2011. Pathways for degradation of lignin in bacteria and fungi. *Nat. Prod. Rep.* 28, 1883–96. doi:10.1039/c1np00042j

Bundhoo, M.A.A.Z., Mohee, R., 2016. Inhibition of dark fermentative bio-hydrogen production: A review. *Int. J. Hydrogen Energy* 41, 6713–6733. doi:10.1016/j.ijhydene.2016.03.057

- Cai, G., Jin, B., Monis, P., Saint, C., 2011. Metabolic flux network and analysis of fermentative hydrogen production. *Biotechnol. Adv.* 29, 375–387. doi:10.1016/j.biotechadv.2011.02.001
- California Energy Commission, 2014. Hydrogen refueling stations funding awards [WWW Document]. Ca.gov. URL hydrogen_refueling_stations_funding_awards
- Calusinska, M., Happe, T., Joris, B., Wilmotte, A., 2010. The surprising diversity of clostridial hydrogenases: A comparative genomic perspective. *Microbiology* 156, 1575–1588. doi:10.1099/mic.0.032771-0
- Caporaso, J.G., Lauber, C.L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. a, Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi:10.1038/ismej.2012.8
- Carriquiry, M.A., Du, X., Timilsina, G.R., 2011. Second generation biofuels: Economics and policies. *Energy Policy* 39, 4222–4234. doi:10.1016/j.enpol.2011.04.036
- Chanakya, H.N., Khuntia, H.K., 2014. Treatment of gray water using anaerobic biofilms created on synthetic and natural fibers. *Process Saf. Environ. Prot.* 92, 186–192. doi:10.1016/j.psep.2012.12.004
- Chaubey, R., Sahu, S., James, O.O., Maity, S., 2013. A review on development of industrial processes and emerging techniques for production of hydrogen from renewable and sustainable sources. *Renew. Sustain. Energy Rev.* 23, 443–462.
- Chen, C.-C., Chuang, Y.-S., Lin, C.-Y., Lay, C.-H., Sen, B., 2012. Thermophilic dark fermentation of untreated rice straw using mixed cultures for hydrogen production. *Int. J. Hydrogen Energy* 37, 15540–15546. doi:10.1016/j.ijhydene.2012.01.036
- Cheng, J., Su, H., Zhou, J., Song, W., Cen, K., 2011. Microwave-assisted alkali pretreatment of rice straw to promote enzymatic hydrolysis and hydrogen production in dark- and photo-fermentation. *Int. J. Hydrogen Energy* 36, 2093–2101. doi:10.1016/j.ijhydene.2010.11.021
- Chu, Y., Wei, Y., Yuan, X., Shi, X., 2011. Bioconversion of wheat stalk to hydrogen by dark fermentation: effect of different mixed microflora on hydrogen yield and cellulose

- solubilisation. *Bioresour. Technol.* 102, 3805–9. doi:10.1016/j.biortech.2010.11.092
- Cisneros-Pérez, C., Carrillo-Reyes, J., Celis, L.B., Alatríste-Mondragón, F., Etchebehere, C., Razo-Flores, E., 2015. Inoculum pretreatment promotes differences in hydrogen production performance in EGSB reactors. *Int. J. Hydrogen Energy* 40, 6329–6339. doi:10.1016/j.ijhydene.2015.03.048
- Clauwaert, P., Aelterman, P., Pham, T.H., De Schampelaire, L., Carballa, M., Rabaey, K., Verstraete, W., 2008. Minimizing losses in bio-electrochemical systems: The road to applications. *Appl. Microbiol. Biotechnol.* 79, 901–913. doi:10.1007/s00253-008-1522-2
- Cook, J., Oreskes, N., Doran, P.T., Anderegg, W.R.L., Verheggen, B., Maibach, E.W., Carlton, J.S., Lewandowsky, S., Skuce, A.G., Green, S.A., Nuccitelli, D., Jacobs, P., Richardson, M., Winkler, B., Painting, R., Rice, K., 2016. Consensus on consensus: a synthesis of consensus estimates on human-caused global warming. *Environ. Res. Lett.* 11, 1–7. doi:10.1088/1748-9326/11/4/048002
- Costa, K.C., Leigh, J.A., 2014. Metabolic versatility in methanogens. *Curr. Opin. Biotechnol.* 29, 70–75. doi:10.1016/j.copbio.2014.02.012
- Cui, M., Shen, J., 2012. Effects of acid and alkaline pretreatments on the biohydrogen production from grass by anaerobic dark fermentation. *Int. J. Hydrogen Energy* 37, 1120–1124. doi:10.1016/j.ijhydene.2011.02.078
- Cusick, R.D., Bryan, B., Parker, D.S., Merrill, M.D., Mehanna, M., Kiely, P.D., Liu, G., Logan, B.E., 2011. Performance of a pilot-scale continuous flow microbial electrolysis cell fed winery wastewater. *Appl. Microbiol. Biotechnol.* 89, 2053–2063. doi:10.1007/s00253-011-3130-9
- Danilenko, A.N., Bogomolov, A.A., Yuryev, V.P., Dianova, V.T., Bogatyrev, A.N., 1993. Effect of the Polymerization Degree, Moisture Content, and Temperature on Kinetics of Hydrolysis of Corn Starch by Alpha-Amylase. *Starch - Stärke* 45, 63–65. doi:10.1002/star.19930450208
- Danko, A.S., Abreu, Â. a., Alves, M.M., 2008. Effect of arabinose concentration on dark fermentation hydrogen production using different mixed cultures. *Int. J. Hydrogen Energy* 33, 4527–4532. doi:10.1016/j.ijhydene.2008.05.060

- Dareioti, M.A., Vavouraki, A.I., Kornaros, M., 2014. Effect of pH on the anaerobic acidogenesis of agroindustrial wastewaters for maximization of bio-hydrogen production: A lab-scale evaluation using batch tests. *Bioresour. Technol.* 162, 218–227. doi:10.1016/j.biortech.2014.03.149
- Das, D., 2001. Hydrogen production by biological processes: a survey of literature. *Int. J. Hydrogen Energy* 26, 13–28. doi:10.1016/S0360-3199(00)00058-6
- Das, D., Dutta, T., Nath, K., Kotay, S.M., Das, A.K., Veziroglu, T.N., 2006. Role of Fe-hydrogenase in biological hydrogen production. *Curr. Sci.* 90, 1627–1637.
- Datar, R., Huang, J., Maness, P., Mohagheghi, A., Czernik, S., Chornet, E., 2007. Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. *Int. J. Hydrogen Energy* 32, 932–939. doi:10.1016/j.ijhydene.2006.09.027
- De Amorim, E.L.C., Sader, L.T., Silva, E.L., 2012. Effect of substrate concentration on dark fermentation hydrogen production using an anaerobic fluidized bed reactor. *Appl. Biochem. Biotechnol.* 166, 1248–1263. doi:10.1007/s12010-011-9511-9
- DeAngelis, K.M., Sharma, D., Varney, R., Simmons, B., Isern, N.G., Markillie, L.M., Nicora, C., Norbeck, A.D., Taylor, R.C., Aldrich, J.T., Robinson, E.W., 2013. Evidence supporting dissimilatory and assimilatory lignin degradation in *Enterobacter lignolyticus* SCF1. *Front. Microbiol.* 4, 1–14. doi:10.3389/fmicb.2013.00280
- De Sá, L.R.V., Cammarota, M.C., De Oliveira, T.C., Oliveira, E.M.M., Matos, A., Ferreira-Leitão, V.S., 2013. Pentoses, hexoses and glycerin as substrates for biohydrogen production: An approach for Brazilian biofuel integration. *Int. J. Hydrogen Energy* 38, 2986–2997. doi:10.1016/j.ijhydene.2012.12.103
- De Sá, L.R. V, De Oliveira, T.C., Dos Santos, T.F., Matos, A., Cammarota, M.C., Oliveira, E.M.M., Ferreira-Leitão, V.S., 2011. Hydrogenase activity monitoring in the fermentative hydrogen production using heat pretreated sludge: A useful approach to evaluate bacterial communities performance. *Int. J. Hydrogen Energy* 36, 7543–7549. doi:10.1016/j.ijhydene.2011.03.119
- Delrue, F., Setier, P.A., Sahut, C., Cournac, L., Roubaud, A., Peltier, G., Froment, A.K., 2012. An economic, sustainability, and energetic model of biodiesel production from

- microalgae. *Bioresour. Technol.* 111, 191–200. doi:10.1016/j.biortech.2012.02.020
- Deo, D., Ozgur, E., Eroglu, I., Gunduz, U., Yucel, M., 2012. Photofermentative Hydrogen Production in Outdoor Conditions, in: Dragica Minic (Ed.), *Hydrogen Energy - Challenges and Perspectives*. InTech, p. 44. doi:10.5772/50390
- Diekert, G., Wohlfarth, G., 1994. Metabolism of homoacetogens. *Antonie Van Leeuwenhoek* 66, 209–221. doi:10.1007/BF00871640
- Ding, H.B., Tan, G.Y.A., Wang, J.Y., 2010. Caproate formation in mixed-culture fermentative hydrogen production. *Bioresour. Technol.* 101, 9550–9559. doi:10.1016/j.biortech.2010.07.056
- Dong, L., Zhenhong, Y., Yongming, S., Xiaoying, K., Yu, Z., 2009. Hydrogen production characteristics of the organic fraction of municipal solid wastes by anaerobic mixed culture fermentation. *Int. J. Hydrogen Energy* 34, 812–820. doi:10.1016/j.ijhydene.2008.11.031
- Duncan, S.H., Louis, P., Flint, H.J., 2007. Cultivable bacterial diversity from the human colon. *Lett. Appl. Microbiol.* 44, 343–350. doi:10.1111/j.1472-765X.2007.02129.x
- Dutta, S., 2014. A review on production, storage of hydrogen and its utilization as an energy resource. *J. Ind. Eng. Chem.* 20, 1148–1156. doi:10.1016/j.jiec.2013.07.037
- Eckermann, E., 2001. *World History of the Automobile*.
- Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi:10.1093/bioinformatics/btr381
- Elsharnouby, O., Hafez, H., Nakhla, G., El Nagggar, M.H., 2013. A critical literature review on biohydrogen production by pure cultures. *Int. J. Hydrogen Energy* 38, 4945–4966. doi:10.1016/j.ijhydene.2013.02.032
- Esquivel-Elizondo, S., Chairez, I., Salgado, E., Aranda, J.S., Baquerizo, G., Garcia-Peña, E.I., 2014. Controlled continuous bio-hydrogen production using different biogas release strategies. *Appl. Biochem. Biotechnol.* 173, 1737–51. doi:10.1007/s12010-014-0961-8
- Estela, R., Luis, J., 2013. Hydrolysis of Biomass Mediated by Cellulases for the Production of Sugars, in: *Sustainable Degradation of Lignocellulosic Biomass - Techniques*,

Applications and Commercialization. InTech. doi:10.5772/53719

European Commission, 2016. European energy projects Funding [WWW Document]. URL <https://ec.europa.eu/energy/en/funding-and-contracts>

Fan, Y.-T., Zhang, Y.-H., Zhang, S.-F., Hou, H.-W., Ren, B.-Z., 2006. Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. *Bioresour. Technol.* 97, 500–505. doi:10.1016/j.biortech.2005.02.049

Fang, T. Zhang, H. Liu, H., 2002. Microbial diversity of a mesophilic hydrogen-producing sludge. *Appl. Microbiol. Biotechnol.* 58, 112–118. doi:10.1007/s00253-001-0865-8

FAO, 2011. Global food losses and food waste - Extent, causes and prevention. Rome.

Favaro, L., Alibardi, L., Lavagnolo, M.C., Casella, S., Basaglia, M., 2013. Effects of inoculum and indigenous microflora on hydrogen production from the organic fraction of municipal solid waste. *Int. J. Hydrogen Energy* 38, 11774–11779. doi:10.1016/j.ijhydene.2013.06.137

Flowers, J.J., Cadkin, T.A., McMahon, K.D., 2013. Seasonal bacterial community dynamics in a full-scale enhanced biological phosphorus removal plant. *Water Res.* 47, 7019–7031. doi:10.1016/j.watres.2013.07.054

FreedomCAR & Fuel Partnership, 2009. Hydrogen production overview of technology options.

Gagnaire, N., Gabrielle, B., Silveira, J. Da, 2006. Une approche économique, énergétique et environnementale du gisement et de la collecte des pailles et d'une utilisation pour les filières éthanol.

Gao, J., Tian, M., 2016. Analysis of over-consumption of natural resources and the ecological trade deficit in China based on ecological footprints. *Ecol. Indic.* 61, 899–904. doi:10.1016/j.ecolind.2015.10.044

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. *Appl. Energy* 144, 73–95. doi:10.1016/j.apenergy.2015.01.045

Gilbert, P., Collier, P.J., Brown, M.R., 1990. Influence of growth rate on susceptibility to

- antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrob. Agents Chemother.* 34, 1865–1868. doi:10.1128/AAC.34.10.1865
- Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Lukasik, R., 2010. Hemicelluloses for fuel ethanol: A review. *Bioresour. Technol.* 101, 4775–4800. doi:10.1016/j.biortech.2010.01.088
- Godon, J., Zumstein, E., Dabert, P., Habouzit, R.I.C., 1997. Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis . *Molecular Microbial Diversity of an Anaerobic Digester as Determined by Small-Subunit rDNA Sequence Analysis. Applied Environ. Microbiol.* 63, 2802–2813. doi:0099-2240
- Guo, X.M., Trably, E., Latrille, E., Carrere, H., Steyer, J.-P., 2014. Predictive and explicative models of fermentative hydrogen production from solid organic waste: Role of butyrate and lactate pathways. *Int. J. Hydrogen Energy* 39, 7476–7485. doi:10.1016/j.ijhydene.2013.08.079
- Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, J.-P., 2010. Hydrogen production from agricultural waste by dark fermentation: A review. *Int. J. Hydrogen Energy* 35, 10660–10673. doi:10.1016/j.ijhydene.2010.03.008
- Gupta, M., Gomez-Flores, M., Nasr, N., Elbeshbishy, E., Hafez, H., Hesham El Nagggar, M., Nakhla, G., 2015. Performance of mesophilic biohydrogen-producing cultures at thermophilic conditions. *Bioresour. Technol.* 192, 741–747. doi:10.1016/j.biortech.2015.06.047
- H2ME, 2015. Hydrogen Mobility Europe – H2ME – is a flagship project giving fuel cell electric vehicle (FCEV) drivers access to the first truly pan-European network of hydrogen refuelling stations [WWW Document]. URL <http://h2me.eu/>
- Hallac, B.B., Ragauskas, A.J., 2011. Analyzing cellulose degree of polymerization and its relevancy to cellulosic ethanol. *Biofuels, Bioprod. Biorefining* 5, 215–225. doi:10.1002/bbb.269
- Hallenbeck, P.C., Abo-Hashesh, M., Ghosh, D., 2012. Strategies for improving biological hydrogen production. *Bioresour. Technol.* 110, 1–9.
- Hamelers, H.V.M., Ter Heijne, A., Sleutels, T.H.J.A., Jeremiasse, A.W., Strik, D.P.B.T.B.,

- Buisman, C.J.N., 2010. New applications and performance of bioelectrochemical systems. *Appl. Microbiol. Biotechnol.* 85, 1673–1685. doi:10.1007/s00253-009-2357-1
- Han, H., Wei, L., Liu, B., Yang, H., Shen, J., 2012. Optimization of biohydrogen production from soybean straw using anaerobic mixed bacteria. *Int. J. Hydrogen Energy* 37, 13200–13208. doi:10.1016/j.ijhydene.2012.03.073
- Harnisch, F., Schröder, U., 2010. From MFC to MXC: chemical and biological cathodes and their potential for microbial bioelectrochemical systems. *Chem. Soc. Rev.* 39, 4433. doi:10.1039/c003068f
- Hawkes, F., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D., 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. *Int. J. Hydrogen Energy* 32, 172–184. doi:10.1016/j.ijhydene.2006.08.014
- Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I., 2002. Sustainable fermentative hydrogen production: Challenges for process optimisation. *Int. J. Hydrogen Energy* 27, 1339–1347. doi:10.1016/S0360-3199(02)00090-3
- Hayashi, M., Hughes, L., 2013. The Fukushima nuclear accident and its effect on global energy security. *Energy Policy* 59, 102–111. doi:10.1016/j.enpol.2012.11.046
- Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* 100, 10–18. doi:10.1016/j.biortech.2008.05.027
- Hisatomi, T., Kubota, J., Domen, K., 2014. Recent advances in semiconductors for photocatalytic and photoelectrochemical water splitting. *Chem. Soc. Rev.* 43, 7520–7535. doi:10.1039/C3CS60378D
- Hoelzle, R.D., Viridis, B., Batstone, D.J., 2014. Regulation mechanisms in mixed and pure culture microbial fermentation. *Biotechnol. Bioeng.* 111, 2139–2154. doi:10.1002/bit.25321
- Hu, Z.-H., Wang, G., Yu, H.-Q., 2004. Anaerobic degradation of cellulose by rumen microorganisms at various pH values. *Biochem. Eng. J.* 21, 59–62. doi:10.1016/j.bej.2004.05.004
- Huang, Y., Zong, W., Yan, X., Wang, R., Hemme, C.L., Zhou, J., Zhou, Z., 2010. Succession of

- the bacterial community and dynamics of hydrogen producers in a hydrogen-producing bioreactor. *Appl. Environ. Microbiol.* 76, 3387–3390. doi:10.1128/AEM.02444-09
- Hung, C.-H., Chang, Y.-T., Chang, Y.-J., 2011. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems – A review. *Bioresour. Technol.* 102, 8437–8444. doi:10.1016/j.biortech.2011.02.084
- Jang, Y.S., Malaviya, A., Lee, J., Im, J.A., Lee, S.Y., Lee, J., Eom, M.H., Cho, J.H., Seung, D.Y., 2013. Metabolic engineering of *Clostridium acetobutylicum* for the enhanced production of isopropanol-butanol-ethanol fuel mixture. *Biotechnol. Prog.* 29, 1083–1088. doi:10.1002/btpr.1733
- Jiang, H., Gadow, S.I., Tanaka, Y., Cheng, J., Li, Y.-Y., 2015. Improved cellulose conversion to bio-hydrogen with thermophilic bacteria and characterization of microbial community in continuous bioreactor. *Biomass and Bioenergy* 75, 57–64. doi:10.1016/j.biombioe.2015.02.010
- Jo, J.H., Jeon, C.O., Lee, D.S., Park, J.M., 2007. Process stability and microbial community structure in anaerobic hydrogen-producing microflora from food waste containing kimchi. *J. Biotechnol.* 131, 300–308. doi:10.1016/j.jbiotec.2007.07.492
- Jo, J.H., Jeon, C.O., Lee, S.Y., Lee, D.S., Park, J.M., 2010. Molecular characterization and homologous overexpression of [FeFe]-hydrogenase in *Clostridium tyrobutyricum* JM1. *Int. J. Hydrogen Energy* 35, 1065–1073. doi:10.1016/j.ijhydene.2009.11.102
- Jobard, M., Pessiot, J., Nouaille, R., Sime-Ngando, T., 2014. Microbial diversity supporting dark fermentation of waste. *Trends Biotechnol.* 32, 549–550. doi:10.1016/j.tibtech.2014.09.005
- Junghare, M., Subudhi, S., Lal, B., 2012. Improvement of hydrogen production under decreased partial pressure by newly isolated alkaline tolerant anaerobe, *Clostridium butyricum* TM-9A: Optimization of process parameters. *Int. J. Hydrogen Energy* 37, 3160–3168. doi:10.1016/j.ijhydene.2011.11.043
- Kahm, M., Kahm, M., Hasenbrink, G., Lichtenberg-Frate, H., Ludwig, J., Kschischo, M., 2010. Grofit: Fitting biological growth curves. *Nat. Preced.* 33, 1–21. doi:10.1038/npre.2010.4508.1

- Kalinci, Y., Hepbasli, A., Dincer, I., 2009. Biomass-based hydrogen production: A review and analysis. *Int. J. Hydrogen Energy* 34, 8799–8817. doi:10.1016/j.ijhydene.2009.08.078
- Kanagawa Prefectural Government, Yokohama, C. of, Kawasaki City, Iwatani Corporation, Toshiba Corporation, Toyota Motor Corporation, 2016. Public-Private Partnership to Test End-to-End Hydrogen Supply Chain.
- Kannaiah Goud, R., Sarkar, O., Venkata Mohan, S., 2014. Regulation of biohydrogen production by heat-shock pretreatment facilitates selective enrichment of *Clostridium* sp. *Int. J. Hydrogen Energy* 39, 7572–7586. doi:10.1016/j.ijhydene.2013.10.046
- Kapdan, I.K., Kargi, F., 2006. Bio-hydrogen production from waste materials. *Enzyme Microb. Technol.* 38, 569–582. doi:10.1016/j.enzmictec.2005.09.015
- Kargi, F., Eren, N.S., Ozmihci, S., 2012. Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations. *Int. J. Hydrogen Energy* 37, 8338–8342. doi:http://dx.doi.org/10.1016/j.ijhydene.2012.02.162
- Karimi, K., Taherzadeh, M.J., 2007. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. *Bioresources* 2, 472–499.
- KIM, D., HAN, S., KIM, S., SHIN, H., 2006. Effect of gas sparging on continuous fermentative hydrogen production. *Int. J. Hydrogen Energy* 31, 2158–2169. doi:10.1016/j.ijhydene.2006.02.012
- Kim, D.-H., Kim, M.-S., 2011. Hydrogenases for biological hydrogen production. *Bioresour. Technol.* 102, 8423–8431. doi:10.1016/j.biortech.2011.02.113
- Kim, Y.J., Lee, H.S., Kim, E.S., Bae, S.S., Lim, J.K., Matsumi, R., Lebedinsky, A. V, Sokolova, T.G., Kozhevnikova, D.A., Cha, S.S., Kim, S.J., Kwon, K.K., Imanaka, T., Atomi, H., Bonch-Osmolovskaya, E.A., Lee, J.H., Kang, S.G., 2010. Formate-driven growth coupled with H₂ production. *Nature* 467, 352–355. doi:10.1038/nature09375
- Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. *Curr. Opin. Biotechnol.* 18, 207–212. doi:10.1016/j.copbio.2007.05.001
- Kleppe, P.J., 1970. Kraft Pulping. *Tappi* 53, 35–47.
- Koch, C., Müller, S., Harms, H., Harnisch, F., 2014. Microbiomes in bioenergy production: From analysis to management. *Curr. Opin. Biotechnol.* 27, 65–72.

doi:10.1016/j.copbio.2013.11.006

Kögel-Knabner, I., 2002. The macromolecular organic composition of Plant and microbial residues as inputs to soil organic matter. *Soil Biol. Biochem.* 34, 139–162. doi:10.1016/S0038-0717(01)00158-4

Koku, H., Eroglu, I., GündPuz, U., Yücel, M., Türker, L., 2002. Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*. *Int. J. Hydrogen Energy* 27, 1315–1329. doi:10.1016/S0360-3199(02)00127-1

Kolb, R., 2014. Short history of biofuels [WWW Document]. *BioEnergy Connect.* URL <http://bioenergyconnection.org/article/short-history-biofuels>

Kotay, S.M., Das, D., 2007. Microbial hydrogen production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge. *Bioresour. Technol.* 98, 1183–1190. doi:10.1016/j.biortech.2006.05.009

Kotay, S.M., Das, D., 2009. Novel dark fermentation involving bioaugmentation with constructed bacterial consortium for enhanced biohydrogen production from pretreated sewage sludge. *Int. J. Hydrogen Energy* 34, 7489–7496. doi:http://dx.doi.org/10.1016/j.ijhydene.2009.05.109

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi:10.1128/AEM.01043-13

Kumar, P., Kumar, P., Barrett, D.M., Barrett, D.M., Delwiche, M.J., Delwiche, M.J., Stroeve, P., Stroeve, P., 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Ind. Eng. Chem. (Analytical Ed.)* 3713–3729. doi:10.1021/ie801542g

Kumar, P., Patel, S.K.S., Lee, J.-K., Kalia, V.C., 2013. Extending the limits of *Bacillus* for novel biotechnological applications. *Biotechnol. Adv.* 31, 1543–1561. doi:10.1016/j.biotechadv.2013.08.007

Kuo, W.-C., Chao, Y.-C., Wang, Y.-C., Cheng, S.-S., 2012. Bioaugmentation Strategies to Improve Cellulolytic and Hydrogen Producing Characteristics in CSTR Intermittent Fed

- with Vegetable Kitchen Waste and Napiergrass. *Energy Procedia* 29, 82–91. doi:10.1016/j.egypro.2012.09.011
- Kyazze, G., Dinsdale, R., Guwy, A.J., Hawkes, F.R., Premier, G.C., Hawkes, D.L., 2007. Performance characteristics of a two-stage dark fermentative system producing hydrogen and methane continuously. *Biotechnol. Bioeng.* 97, 759–770. doi:10.1002/bit.21297
- Kyazze, G., Dinsdale, R., Hawkes, F.R., Guwy, a J., Premier, G.C., Donnison, I.S., 2008. Direct fermentation of fodder maize, chicory fructans and perennial ryegrass to hydrogen using mixed microflora. *Bioresour. Technol.* 99, 8833–9. doi:10.1016/j.biortech.2008.04.047
- Lakaniemi, A.-M., Koskinen, P.E.P., Nevatalo, L.M., Kaksonen, A.H., Puhakka, J. a., 2011. Biogenic hydrogen and methane production from reed canary grass. *Biomass and Bioenergy* 35, 773–780. doi:10.1016/j.biombioe.2010.10.032
- Lakaniemi, A.-M., Tuovinen, O.H., Puhakka, J.A., 2013. Anaerobic conversion of microalgal biomass to sustainable energy carriers – A review. *Bioresour. Technol.* 135, 222–231. doi:10.1016/j.biortech.2012.08.096
- Larqué, E., Zamora, S., Gil, A., 2001. Dietary trans fatty acids in early life: a review. *Early Hum. Dev.* 65, S31–S41. doi:10.1016/S0378-3782(01)00201-8
- Latrille, E., Trably, E., Larroche, C., 2011. Production de biohydrogène : voie fermentaire sombre. *Tech. l'ingénieur BIO3351*, 1–19.
- Le Cao, K.-A., Gonzalez, I., Dejean, S., 2015. Omics Data Integration Project.
- Lee, D.J., Show, K.Y., Su, A., 2011. Dark fermentation on biohydrogen production: Pure culture. *Bioresour. Technol.* 102, 8393–8402. doi:10.1016/j.biortech.2011.03.041
- Lee, M.-J., Song, J.-H., Hwang, S.-J., 2009. Effects of acid pre-treatment on bio-hydrogen production and microbial communities during dark fermentation. *Bioresour. Technol.* 100, 1491–1493. doi:http://dx.doi.org/10.1016/j.biortech.2008.08.019
- Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. *Oecologia* 129, 271–280. doi:10.1007/s004420100716
- Li, C., Fang, H.H.P., 2007. Fermentative Hydrogen Production From Wastewater and Solid

- Wastes by Mixed Cultures. *Crit. Rev. Environ. Sci. Technol.* 37, 1–39. doi:10.1080/10643380600729071
- Li, D., Chen, H., 2007. Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation. *Int. J. Hydrogen Energy* 32, 1742–1748. doi:10.1016/j.ijhydene.2006.12.011
- Liao, L., Pasternak, I., 2009. A review of airship structural research and development. *Prog. Aerosp. Sci.* 45, 83–96. doi:10.1016/j.paerosci.2009.03.001
- Lin, C.-Y., Miyake, J., Susilaningih, D., Lay, C.-H., Sen, B., Chu, C.-Y., Kumar, G., Chen, C.-C., Chang, J.-S., 2012. Fermentative hydrogen production from wastewaters: A review and prognosis. *Int. J. Hydrogen Energy* 37, 15632–15642.
- Lin, P.-Y., Whang, L.-M., Wu, Y.-R., Ren, W.-J., Hsiao, C.-J., Li, S.-L., Chang, J.-S., 2007. Biological hydrogen production of the genus *Clostridium*: Metabolic study and mathematical model simulation. *Int. J. Hydrogen Energy* 32, 1728–1735. doi:10.1016/j.ijhydene.2006.12.009
- Link, H., Fuhrer, T., Gerosa, L., Zamboni, N., Sauer, U., 2015. Real-time metabolome profiling of the metabolic switch between starvation and growth. *Nat Meth* 12, 1091–1097.
- Liu, H., Grot, S., Logan, B.E., 2005. Electrochemically Assisted Microbial Production of Hydrogen from Acetate. *Environ. Sci. Technol.* 39, 4317–4320. doi:10.1021/es050244p
- Liu, W., Huang, S., Zhou, A., Zhou, G., Ren, N., Wang, A., Zhuang, G., 2012. Hydrogen generation in microbial electrolysis cell feeding with fermentation liquid of waste activated sludge. *Int. J. Hydrogen Energy* 37, 13859–13864. doi:10.1016/j.ijhydene.2012.04.090
- Liu, X., Zhu, Y., Yang, S.T., 2006. Construction and characterization of ack deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. *Biotechnol. Prog.* 22, 1265–1275. doi:10.1021/bp060082g
- Logan, B.E., 2010. Scaling up microbial fuel cells and other bioelectrochemical systems. *Appl. Microbiol. Biotechnol.* 85, 1665–1671. doi:10.1007/s00253-009-2378-9
- Luo, G., Karakashev, D., Xie, L., Zhou, Q., Angelidaki, I., 2011. Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations:

- Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol. Bioeng.* 108, 1816–1827. doi:10.1002/bit.23122
- Macfarlane, G.T., Hay, S., Macfarlane, S., Gibson, G.R., 1990. Effect of different carbohydrates on growth, polysaccharidase and glycosidase production by *Bacteroides ovatus*, in batch and continuous culture. *J. Appl. Bacteriol.* 68, 179–187. doi:10.1111/j.1365-2672.1990.tb02564.x
- Maintinguer, S.I., Sakamoto, I.K., T. Adorno, M.A., A. Varesche, M.B., 2015. Bacterial diversity from environmental sample applied to bio-hydrogen production. *Int. J. Hydrogen Energy* 40, 3180–3190. doi:10.1016/j.ijhydene.2014.12.118
- Mäkinen, A.E., Nissilä, M.E., Puhakka, J.A., 2012. Dark fermentative hydrogen production from xylose by a hot spring enrichment culture. *Int. J. Hydrogen Energy* 37, 12234–12240. doi:10.1016/j.ijhydene.2012.05.158
- MANISH, S., BANERJEE, R., 2008. Comparison of biohydrogen production processes. *Int. J. Hydrogen Energy* 33, 279–286. doi:10.1016/j.ijhydene.2007.07.026
- Marbán, G., Valdés-Solís, T., 2007. Towards the hydrogen economy? *Int. J. Hydrogen Energy* 32, 1625–1637. doi:10.1016/j.ijhydene.2006.12.017
- Margulis, L., Olendzenski, L., Afzelius, B.A., 1990. Endospore-forming filamentous bacteria symbiotic in termites: ultrastructure and growth in culture of *Arthromitus*. *Symbiosis* 8, 95–116.
- Marone, A., Izzo, G., Mentuccia, L., Massini, G., Paganin, P., Rosa, S., Varrone, C., Signorini, A., 2014. Vegetable waste as substrate and source of suitable microflora for bio-hydrogen production. *Renew. Energy* 68, 6–13. doi:10.1016/j.renene.2014.01.013
- McMurdie, P.J., Holmes, S., 2014. Handling and analysis of high-throughput microbiome census data. Description: phyloseq provides a set of classes and tools; version 1.10.0.
- Megret, O., Hubert, L., Calbry, M., Trably, E., Carrere, H., Garcia-Bernet, D., Bernet, N., 2015. Production d'hydrogène à partir de déchets, état de l'art et potentiel d'émergence. doi:Etude RECORD n°13-0239/1A
- Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Prog. Energy Combust. Sci.* 38, 522–550.

doi:10.1016/j.pecs.2012.02.002

- Midilli, A., Ay, M., Dincer, I., Rosen, M. a., 2005. On hydrogen and hydrogen energy strategies. *Renew. Sustain. Energy Rev.* 9, 255–271. doi:10.1016/j.rser.2004.05.003
- Mizrahi-Man, O., Davenport, E.R., Gilad, Y., 2013. Taxonomic Classification of Bacterial 16S rRNA Genes Using Short Sequencing Reads: Evaluation of Effective Study Designs. *PLoS One* 8, e53608. doi:10.1371/journal.pone.0053608
- Mohana, S., Shah, A., Divecha, J., Madamwar, D., 2008. Xylanase production by *Burkholderia* sp. DMAX strain under solid state fermentation using distillery spent wash. *Bioresour. Technol.* 99, 7553–7564. doi:10.1016/j.biortech.2008.02.009
- Monlau, F., Barakat, A., Steyer, J.P., Carrere, H., 2012a. Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks. *Bioresour. Technol.* 120, 241–7. doi:10.1016/j.biortech.2012.06.040
- Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrère, H., 2013a. Lignocellulosic Materials Into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment. *Crit. Rev. Environ. Sci. Technol.* 43, 260–322. doi:10.1080/10643389.2011.604258
- Monlau, F., Sambusiti, C., Barakat, A., Guo, X.M., Latrille, E., Trably, E., Steyer, J.-P., Carrere, H., 2012b. Predictive Models of Biohydrogen and Biomethane Production Based on the Compositional and Structural Features of Lignocellulosic Materials. *Environ. Sci. Technol.* 46, 12217–12225. doi:10.1021/es303132t
- Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.-P., Carrère, H., 2014. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnol. Adv.* 32, 934–51. doi:10.1016/j.biotechadv.2014.04.007
- Monlau, F., Trably, E., Barakat, A., Hamelin, J., Steyer, J.-P., Carrere, H., 2013b. Two-Stage Alkaline-Enzymatic Pretreatments To Enhance Biohydrogen Production from Sunflower Stalks. *Environ. Sci. Technol.* doi:10.1021/es402863v
- Moscoviz, R., Toledo-Alarcón, J., Trably, E., Bernet, N., 2016a. Electro-Fermentation: How To Drive Fermentation Using Electrochemical Systems. *Trends Biotechnol.* xx, 1–10.

doi:10.1016/j.tibtech.2016.04.009

Moscoviz, R., Trably, E., Bernet, N., 2016b. Consistent 1,3-propanediol production from glycerol in mixed culture fermentation over a wide range of pH. *Biotechnol. Biofuels* 9, 32. doi:10.1186/s13068-016-0447-8

Motte, J., 2013. Solid-state anaerobic digestion of lignocellulosic residues : dynamical study of the relationship between process parameters, substrate characteristics and microbial ecosystem. Montpellier 2.

Motte, J.C., Escudié, R., Hamelin, J., Steyer, J.P., Bernet, N., Delgenes, J.P., Dumas, C., 2015. Substrate milling pretreatment as a key parameter for Solid-State Anaerobic Digestion optimization. *Bioresour. Technol.* 173, 185–192. doi:10.1016/j.biortech.2014.09.015

Nasirian, N., Almassi, M., Minaei, S., Widmann, R., 2011. Development of a method for biohydrogen production from wheat straw by dark fermentation. *Int. J. Hydrogen Energy* 36, 411–420. doi:10.1016/j.ijhydene.2010.09.073

Navarro-Díaz, M., Valdez-Vazquez, I., Escalante, A.E., 2016. Ecological perspectives of hydrogen fermentation by microbial consortia: What we have learned and the way forward. *Int. J. Hydrogen Energy* 41, 17297–17308. doi:10.1016/j.ijhydene.2016.08.027

Ntaikou, I., Antonopoulou, G., Lyberatos, G., 2010. Biohydrogen Production from Biomass and Wastes via Dark Fermentation: A Review. *Waste and Biomass Valorization* 1, 21–39. doi:10.1007/s12649-009-9001-2

Oey, M., Sawyer, A.L., Ross, I.L., Hankamer, B., 2016. Challenges and opportunities for hydrogen production from microalgae. *Plant Biotechnol. J.* 14, 1487–1499. doi:10.1111/pbi.12516

Okamoto, M., Miyahara, T., Mizuno, O., Noike, T., 2000. Biological hydrogen potential of materials characteristic of the organic fraction of municipal solid wastes. *Water Sci. Technol.* 41, 25–32.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O’Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2016. *vegan: Community Ecology Package*. R package version 2.3-3. <http://CRAN.R-project.org/package=vegan>.

Organisation for Economic co-operation and Development, O., 2001. Glossary of

- Environment Statistics, studies in methods [WWW Document]. URL <https://stats.oecd.org/glossary/detail.asp?ID=77>
- Ozkan, L., Erguder, T.H., Demirer, G.N., 2011. Effects of pretreatment methods on solubilization of beet-pulp and bio-hydrogen production yield. *Int. J. Hydrogen Energy* 36, 382–389. doi:10.1016/j.ijhydene.2010.10.006
- Oztekin, R., Kapdan, I.K., Kargi, F., Argun, H., 2008. Optimization of media composition for hydrogen gas production from hydrolyzed wheat starch by dark fermentation. *Int. J. Hydrogen Energy* 33, 4083–4090. doi:http://dx.doi.org/10.1016/j.ijhydene.2008.05.052
- Pakarinen, O., 2008. Batch dark fermentative hydrogen production from grass silage: The effect of inoculum, pH, temperature and VS ratio. *Int. J. Hydrogen Energy* 33, 594–601. doi:10.1016/j.ijhydene.2007.10.008
- Pakarinen, O.M., Tähti, H.P., Rintala, J. a., 2009. One-stage H₂ and CH₄ and two-stage H₂+CH₄ production from grass silage and from solid and liquid fractions of NaOH pre-treated grass silage. *Biomass and Bioenergy* 33, 1419–1427. doi:10.1016/j.biombioe.2009.06.006
- Paliy, O., Shankar, V., 2016. Application of multivariate statistical techniques in microbial ecology. *Mol. Ecol.* 25, 1032–1057. doi:10.1111/mec.13536
- Pan, C., Fan, Y., Hou, H., 2008. Fermentative Production of Hydrogen from Wheat Bran by Mixed Anaerobic Cultures. *Ind. Eng. Chem. Res.* 47, 5812–5818. doi:10.1021/ie701789c
- Pandey, M.P., Kim, C.S., 2011. Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chem. Eng. Technol.* 34, 29–41. doi:10.1002/ceat.201000270
- Pant, D., Singh, A., Van Bogaert, G., Irving Olsen, S., Singh Nigam, P., Diels, L., Vanbroekhoven, K., 2012. Bioelectrochemical systems (BES) for sustainable energy production and product recovery from organic wastes and industrial wastewaters. *RSC Adv.* 2, 1248–1263. doi:10.1039/C1RA00839K
- Parthasarathy, P., Narayanan, K.S., 2014. Hydrogen production from steam gasification of biomass: Influence of process parameters on hydrogen yield - A review. *Renew. Energy* 66, 570–579. doi:10.1016/j.renene.2013.12.025 Review

- Patel, S.K.S., Kumar, P., Mehariya, S., Purohit, H.J., Lee, J.K., Kalia, V.C., 2014. Enhancement in hydrogen production by co-cultures of *Bacillus* and *Enterobacter*. *Int. J. Hydrogen Energy* 39, 14663–14668. doi:10.1016/j.ijhydene.2014.07.084
- Pereira, I.A.C., Ramos, A.R., Grein, F., Marques, M.C., da Silva, S.M., Venceslau, S.S., 2011. A Comparative Genomic Analysis of Energy Metabolism in Sulfate Reducing Bacteria and Archaea. *Front. Microbiol.* 2, 1–22. doi:10.3389/fmicb.2011.00069
- Perkins, C., Weimer, A.W., 2004. Likely near-term solar-thermal water splitting technologies. *Int. J. Hydrogen Energy* 29, 1587–1599. doi:10.1016/j.ijhydene.2004.02.019
- Philippidis, G.P., Smith, T.K., Wyman, C.E., 1993. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. *Biotechnol. Bioeng.* 41, 846–853. doi:10.1002/bit.260410903
- Pieraerts, J., 2016. H₂ : The path to a sustainable society, in: World Hydrogen Energy Conference.
- Pires, J.C.M., Alvim-Ferraz, M.C.M., Martins, F.G., Simões, M., 2012. Carbon dioxide capture from flue gases using microalgae: Engineering aspects and biorefinery concept. *Renew. Sustain. Energy Rev.* 16, 3043–3053. doi:10.1016/j.rser.2012.02.055
- Piscina, P.R. de la, Homs, N., 2008. Use of biofuels to produce hydrogen (reformation processes). *Chem. Soc. Rev.* 37, 2459. doi:10.1039/b712181b
- Playne, M.J., 1984. Increased digestibility of bagasses by pretreatment with alkalis and steam explosion. *Biotechnol. Bioeng.* 26, 426–433. doi:10.1002/bit.260260505
- Poirot, R., 2015. Une première : cinq taxi roulent à l'hydrogène dans Paris. *Libération*.
- Porwal, S., Kumar, T., Lal, S., Rani, A., Kumar, S., Cheema, S., Purohit, H.J., Sharma, R., Singh Patel, S.K., Kalia, V.C., 2008. Hydrogen and polyhydroxybutyrate producing abilities of microbes from diverse habitats by dark fermentative process. *Bioresour. Technol.* 99, 5444–5451. doi:10.1016/j.biortech.2007.11.011
- Poudyal, R.S., Tiwari, I., Koirala, A.R., Masukawa, H., Inoue, K., Tomo, T., Najafpour, M.M., Allakhverdiev, S.I., Veziroğlu, T.N., 2015. Hydrogen production using photobiological methods, in: *Compendium of Hydrogen Energy*. Elsevier, pp. 289–317. doi:10.1016/B978-1-78242-361-4.00010-8

- Prakasham, R.S., Brahmaiah, P., Sathish, T., Sambasiva Rao, K.R.S., 2009. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *Int. J. Hydrogen Energy* 34, 9354–9361. doi:10.1016/j.ijhydene.2009.09.104
- Qiu, C., Zheng, Y., Zheng, J., Liu, Y., Xie, C., Sun, L., 2016. Mesophilic and Thermophilic Biohydrogen Production from Xylose at Various Initial pH and Substrate Concentrations with Microflora Community Analysis. *Energy & Fuels* 30, 1013–1019. doi:10.1021/acs.energyfuels.5b02143
- Quéméneur, M., Bittel, M., Trably, E., Dumas, C., Fourage, L., Ravot, G., Steyer, J.-P., Carrère, H., 2012a. Effect of enzyme addition on fermentative hydrogen production from wheat straw. *Int. J. Hydrogen Energy* 37, 10639–10647. doi:10.1016/j.ijhydene.2012.04.083
- Quéméneur, M., Hamelin, J., Barakat, A., Steyer, J.-P., Carrère, H., Trably, E., 2012b. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. *Int. J. Hydrogen Energy* 37, 3150–3159. doi:10.1016/j.ijhydene.2011.11.033
- Quéméneur, M., Hamelin, J., Benomar, S., Guidici-Orticoni, M.-T., Latrille, E., Steyer, J.-P., Trably, E., 2011a. Changes in hydrogenase genetic diversity and proteomic patterns in mixed-culture dark fermentation of mono-, di- and tri-saccharides. *Int. J. Hydrogen Energy* 36, 11654–11665. doi:10.1016/j.ijhydene.2011.06.010
- Quéméneur, M., Hamelin, J., Latrille, E., Steyer, J.-P., Trably, E., 2011b. Functional versus phylogenetic fingerprint analyses for monitoring hydrogen-producing bacterial populations in dark fermentation cultures. *Int. J. Hydrogen Energy* 36, 3870–3879. doi:10.1016/j.ijhydene.2010.12.100
- Rafrafi, Y., 2012. Impact des facteurs biotiques sur le réseau métabolique des écosystèmes producteurs d'hydrogène par voie fermentaire en culture mixte.
- Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., Giudici-Orticoni, M.-T., Steyer, J.-P., 2013. Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen. *Int. J. Hydrogen Energy* 38, 4975–4985. doi:10.1016/j.ijhydene.2013.02.008
- Rajhi, H., Conthe, M., Puyol, D., Díaz, E., Sanz, J.L., 2013. Dark fermentation: isolation and characterization of hydrogen-producing strains from sludges. *Int. Microbiol.* 16, 53–62.

doi:10.2436/20.1501.01.180

- Ramachandran, R., Menon, R.K., 1998. An overview of industrial uses of hydrogen. *Int. J. Hydrogen Energy* 23, 593–598. doi:10.1016/S0360-3199(97)00112-2
- Ramette, A., 2007. Multivariate analyses in microbial ecology. *FEMS Microbiol. Ecol.* 62, 142–160. doi:10.1111/j.1574-6941.2007.00375.x
- REN, N., LI, J., LI, B., WANG, Y., LIU, S., 2006. Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *Int. J. Hydrogen Energy* 31, 2147–2157. doi:10.1016/j.ijhydene.2006.02.011
- Ren, N., Wang, A., Cao, G., Xu, J., Gao, L., 2009. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. *Biotechnol. Adv.* 27, 1051–60. doi:10.1016/j.biotechadv.2009.05.007
- Ren, N., Xing, D., Rittmann, B.E., Zhao, L., Xie, T., Zhao, X., 2007. Microbial community structure of ethanol type fermentation in bio-hydrogen production. *Environ. Microbiol.* 9, 1112–25. doi:10.1111/j.1462-2920.2006.01234.x
- Ringnér, M., 2008. What is principal component analysis? *Nat. Biotechnol.* 26, 303–304. doi:10.1038/nbt0308-303
- Romano, S., Paganin, P., Varrone, C., Tabacchioni, S., Chiarini, L., 2014. Dynamics of hydrogen-producing bacteria in a repeated batch fermentation process using lake sediment as inoculum. *Arch. Microbiol.* 196, 97–107. doi:10.1007/s00203-013-0947-x
- Rouches, E., Herpoël-Gimbert, I., Steyer, J.P., Carrere, H., 2016. Improvement of anaerobic degradation by white-rot fungi pretreatment of lignocellulosic biomass: A review. *Renew. Sustain. Energy Rev.* 59, 179–198. doi:10.1016/j.rser.2015.12.317
- ROZENDAL, R., HAMELERS, H., EUVERINK, G., METZ, S., BUISMAN, C., 2006. Principle and perspectives of hydrogen production through biocatalyzed electrolysis. *Int. J. Hydrogen Energy* 31, 1632–1640. doi:10.1016/j.ijhydene.2005.12.006
- Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *Int. J. Hydrogen Energy* 38, 13172–13191. doi:10.1016/j.ijhydene.2013.07.122
- Saha, B.C., 2003. Hemicellulose bioconversion. *J. Ind. Microbiol. Biotechnol.* 30, 279–291.

doi:10.1007/s10295-003-0049-x

- Saint-Amans, S., Girbal, L., Andrade, J., Ahrens, K., Soucaille, P., 2001. Regulation of Carbon and Electron Flow in *Clostridium butyricum* VPI 3266 Grown on Glucose-Glycerol Mixtures. *J. Bacteriol.* 183, 1748–1754. doi:10.1128/JB.183.5.1748-1754.2001
- Sambusiti, C., Monlau, F., Ficara, E., Carrère, H., Malpei, F., 2013. A comparison of different pre-treatments to increase methane production from two agricultural substrates. *Appl. Energy* 104, 62–70. doi:10.1016/j.apenergy.2012.10.060
- Scheller, H.V., Ulvskov, P., 2010. Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289. doi:10.1146/annurev-arplant-042809-112315
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi:10.1128/AEM.01541-09
- Schwarz, W.H., 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Appl. Microbiol. Biotechnol.* 56, 634–649. doi:10.1007/s002530100710
- Shaishav, S., Satyendra, T., Singh, R., 2013. Biohydrogen from Algae: Fuel of the Future. *Int. Res. J. Environ. Sci.* 2, 44–47.
- Shi, X.-X., Song, H.-C., Wang, C.-R., Tang, R.-S., Huang, Z., Gao, T., Xie, J., 2009. Enhanced biohydrogen production from sweet sorghum stalk with alkalization pretreatment by mixed anaerobic cultures. *Int. J. Energy Res.* 34, n/a-n/a. doi:10.1002/er.1570
- Si, B., Li, J., Li, B., Zhu, Z., Shen, R., Zhang, Y., Liu, Z., 2015. The role of hydraulic retention time on controlling methanogenesis and homoacetogenesis in biohydrogen production using upflow anaerobic sludge blanket (UASB) reactor and packed bed reactor (PBR). *Int. J. Hydrogen Energy* 40, 11414–11421. doi:10.1016/j.ijhydene.2015.04.035
- Sikora, A., Baszczyk, M., Jurkowski, M., Zielenkiewicz, U., 2013. Lactic Acid Bacteria in Hydrogen-Producing Consortia: On Purpose or by Coincidence?, in: *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*. InTech, pp. 487–514. doi:10.5772/50364
- Singh, L., Wahid, Z.A., 2015. Methods for enhancing bio-hydrogen production from biological

- process: A review. *J. Ind. Eng. Chem.* 21, 70–80. doi:10.1016/j.jiec.2014.05.035
- Singh, U.B., Ahluwalia, A.S., 2013. Microalgae: a promising tool for carbon sequestration. *Mitig. Adapt. Strateg. Glob. Chang.* 18, 73–95. doi:10.1007/s11027-012-9393-3
- Skoog, K., Hahn-Hägerdal, B., 1988. Xylose fermentation. *Enzyme Microb. Technol.* 10, 66–80. doi:10.1016/0141-0229(88)90001-4
- Snel, J., Heinen, P.P., Blok, H.J., Carman, R.J., Duncan, a J., Allen, P.C., Collins, M.D., 1995. Comparison of 16s rRNA Sequences of Segmented Filamentous Bacteria Isolated from Mice, Rats, and Chickens and Proposal of “Candidatus Arthromitus.” *Int. J. Syst. Bacteriol.* 45, 780–2.
- Song, W., Cheng, J., Zhao, J., Zhang, C., Zhou, J., Cen, K., 2016. Enhancing hydrogen production of *Enterobacter aerogenes* by heterologous expression of hydrogenase genes originated from *Synechocystis* sp. *Bioresour. Technol.* 216, 976–980. doi:10.1016/j.biortech.2016.06.044
- Stiebritz, M.T., Reiher, M., 2012. Hydrogenases and oxygen. *Chem. Sci.* 3, 1739. doi:10.1039/c2sc01112c
- Stülke, J., Hillen, W., 2000. Regulation of Carbon Catabolism in *Bacillus* Species. *Annu. Rev. Microbiol.* 54, 849–880. doi:10.1146/annurev.micro.54.1.849
- Su, H., Cheng, J., Zhou, J., Song, W., Cen, K., 2009. Improving hydrogen production from cassava starch by combination of dark and photo fermentation. *Int. J. Hydrogen Energy* 34, 1780–1786. doi:10.1016/j.ijhydene.2008.12.045
- Sun, D.-L., Jiang, X., Wu, Q.L., Zhou, N.-Y., 2013. Intragenomic Heterogeneity of 16S rRNA Genes Causes Overestimation of Prokaryotic Diversity. *Appl. Environ. Microbiol.* 79, 5962–5969. doi:10.1128/AEM.01282-13
- Tajima, Y., Yamamoto, Y., Fukui, K., Nishio, Y., Hashiguchi, K., Usuda, Y., Sode, K., 2015. Effects of eliminating pyruvate node pathways and of coexpression of heterogeneous carboxylation enzymes on succinate production by *Enterobacter aerogenes*. *Appl. Environ. Microbiol.* 81, 929–937. doi:10.1128/AEM.03213-14
- Talebnia, F., Karakashev, D., Angelidaki, I., 2010. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresour. Technol.* 101,

4744–4753. doi:10.1016/j.biortech.2009.11.080

Temudo, M.F., Mato, T., Kleerebezem, R., Van Loosdrecht, M.C.M., 2009. Xylose anaerobic conversion by open-mixed cultures. *Appl. Microbiol. Biotechnol.* 82, 231–239. doi:10.1007/s00253-008-1749-y

Thomas, L., Joseph, A., Gottumukkala, L.D., 2014. Xylanase and cellulase systems of *Clostridium* sp.: An insight on molecular approaches for strain improvement. *Bioresour. Technol.* 158, 343–350. doi:10.1016/j.biortech.2014.01.140

Thompson, C.L., Vier, R., Mikaelyan, A., Wienemann, T., Brune, A., 2012. “Candidatus *Arthromitus*” revised: Segmented filamentous bacteria in arthropod guts are members of Lachnospiraceae. *Environ. Microbiol.* 14, 1454–1465. doi:10.1111/j.1462-2920.2012.02731.x

Toklu, E., Coskun Avcı, A., Kaygusuz, K., Gur, M., 2016. A research on hydrogen production from industrial waste heat by thermal water splitting. *Int. J. Hydrogen Energy* 41, 10071–10079. doi:10.1016/j.ijhydene.2015.12.131

Tokyo metropolitan Government, B. of E., 2016. Tokyo Environmental Master Plan. Tokyo.

Toth, G., Szigeti, C., 2016. The historical ecological footprint: From over-population to over-consumption. *Ecol. Indic.* 60, 283–291. doi:10.1016/j.ecolind.2015.06.040

Turon, V., Trably, E., Fouilland, E., Steyer, J.-P., 2016. Potentialities of dark fermentation effluent as substrates for microalgae growth: A review. *Process Biochem.* doi:10.1016/j.procbio.2016.03.018

U.S. Department of Energy, 2016. Hydrogen Energy California Project [WWW Document]. Energy.gov. URL <http://energy.gov/fe/hydrogen-energy-california-project>

U.S. DRIVE Partnership., 2013. Hydrogen Production Technical Team Roadmap.

UNFCCC. Conference of the Parties (COP), 2015. Adoption of the Paris Agreement. Proposal by the President., in: Paris Climate Change Conference - November 2015, COP 21. Paris, p. 32. doi:FCCC/CP/2015/L.9/Rev.1

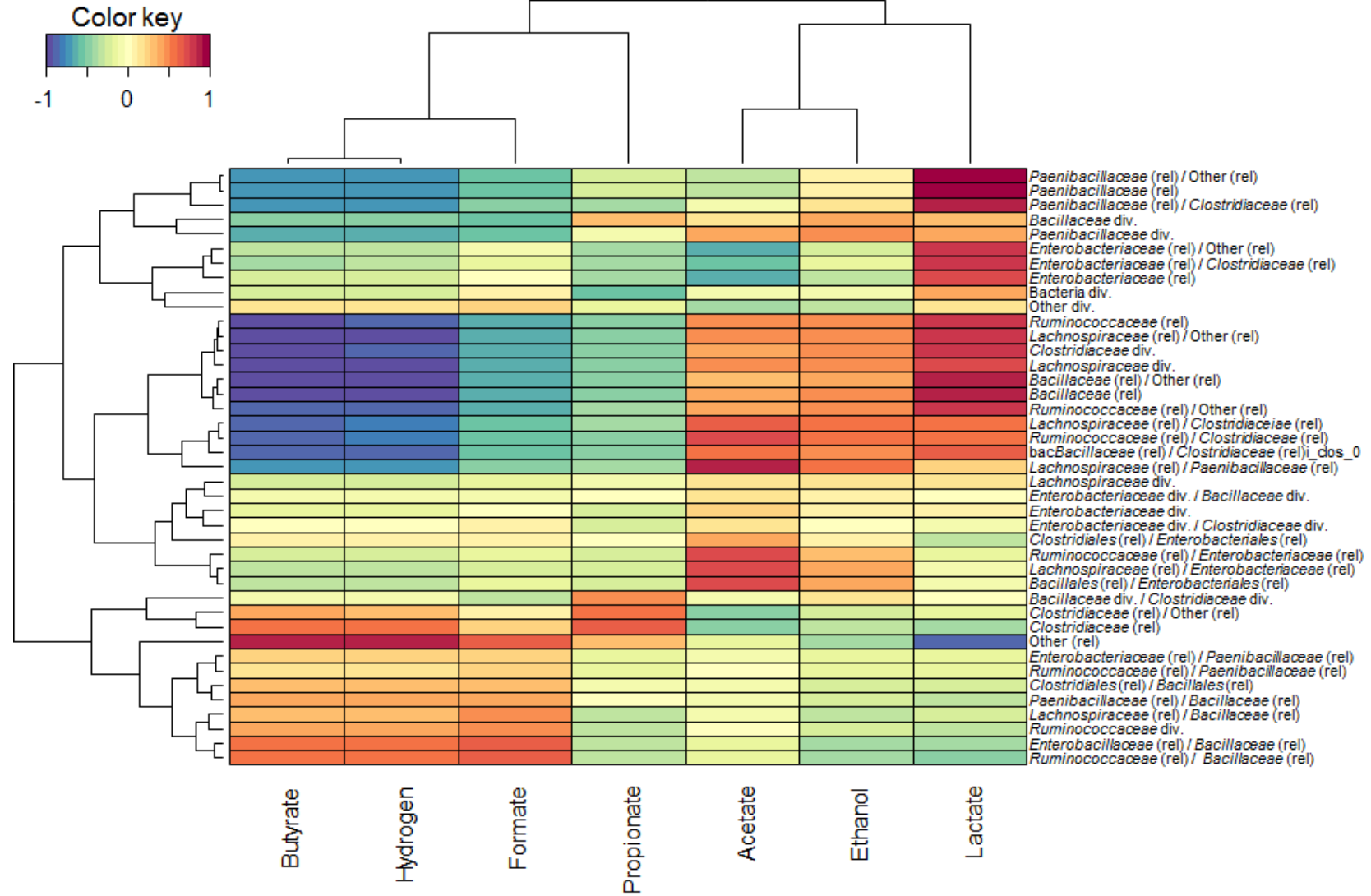
Uyar, B., Kars, G., Yücel, M., Gündüz, U., Eroglu, I., 2012. Hydrogen production via Photofermentation, in: State of the Art and Progress in Production of Biohydrogen. pp. 54–77.

- Vaezi, M., Passandideh-Fard, M., Moghiman, M., Charmchi, M., 2012. On a methodology for selecting biomass materials for gasification purposes. *Fuel Process. Technol.* 98, 74–81. doi:10.1016/j.fuproc.2012.01.026
- van Aarle, I.M., Perimenis, A., Lima-Ramos, J., de Hults, E., George, I.F., Gerin, P.A., 2015. Mixed inoculum origin and lignocellulosic substrate type both influence the production of volatile fatty acids during acidogenic fermentation. *Biochem. Eng. J.* 103, 242–249. doi:10.1016/j.bej.2015.07.016
- Van-Dal, É.S., Bouallou, C., 2013. Design and simulation of a methanol production plant from CO₂ hydrogenation. *J. Clean. Prod.* 57, 38–45. doi:10.1016/j.jclepro.2013.06.008
- Vassileva, I., Dahlquist, E., Campillo, J., 2016. The Citizens' Role in Energy Smart City Development. *Energy Procedia* 88, 200–204. doi:10.1016/j.egypro.2016.06.055
- Vassilev, S. V., Baxter, D., Andersen, L.K., Vassileva, C.G., Morgan, T.J., 2012. An overview of the organic and inorganic phase composition of biomass. *Fuel* 94, 1–33. doi:10.1016/j.fuel.2011.09.030
- Venkata Mohan, S., Lalit Babu, V., Sarma, P.N., 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. *Bioresour. Technol.* 99, 59–67. doi:10.1016/j.biortech.2006.12.004
- Venkata Mohan, S., Mohanakrishna, G., Goud, R.K., Sarma, P.N., 2009. Acidogenic fermentation of vegetable based market waste to harness biohydrogen with simultaneous stabilization. *Bioresour. Technol.* 100, 3061–8. doi:10.1016/j.biortech.2008.12.059
- Verbecke, F., Vesly, B., 2013. Safety strategy for the first deployment of a hydrogen-based green public building in France. *Int. J. Hydrogen Energy* 38, 8053–8060. doi:10.1016/j.ijhydene.2013.03.019
- Viala, M., 2013. Projet JANUS: Développer et mutualiser un ensemble de chaîne hydrogène pour permettre l'autonomie énergétique et le secours des bâtiments, in: *Journées H2 Dans Les Territoires*. Labessière-Candeil, France, p. 4.
- Vonabendroth, G., Stripp, S., Silakov, A., Croux, C., Soucaille, P., Girbal, L., Happe, T., 2008.

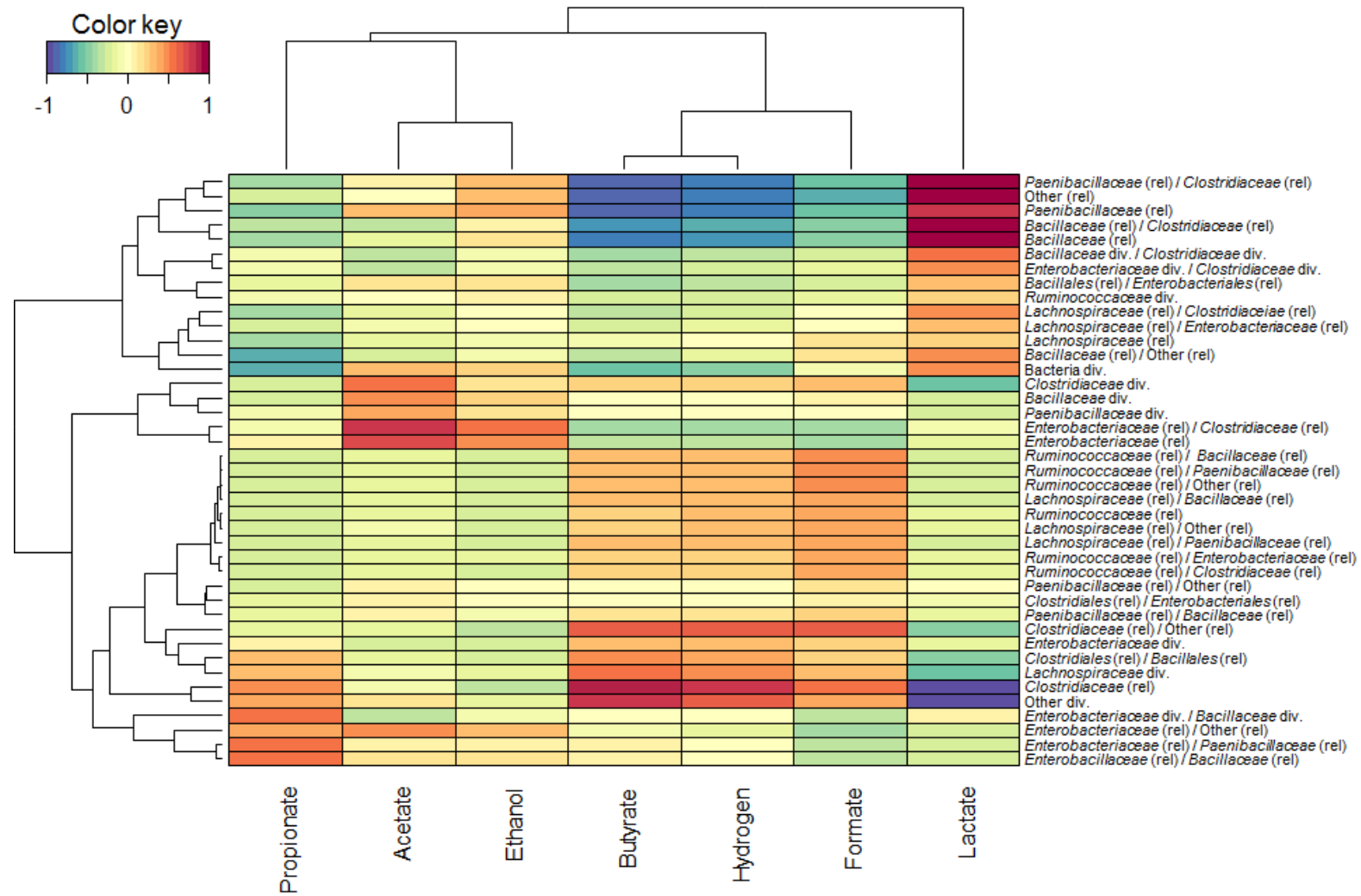
- Optimized over-expression of [FeFe] hydrogenases with high specific activity in *Clostridium acetobutylicum*. *Int. J. Hydrogen Energy* 33, 6076–6081. doi:10.1016/j.ijhydene.2008.07.122
- Vu, V.Q., 2011. ggbiplot: A ggplot2 based biplot. R package version 0.55. <http://github.com/vqv/ggbiplot>.
- Wang, a, 2008. Bioaugmented hydrogen production from microcrystalline cellulose using co-culture—*Clostridium acetobutylicum* X9X9 and *Ethanoigenens harbinense* B49B49. *Int. J. Hydrogen Energy* 33, 912–917. doi:10.1016/j.ijhydene.2007.10.017
- Wang, J., Yu, W., Xu, L., Wang, S., Yan, Y., 2013. Effects of increasing the NAD(H) pool on hydrogen production and metabolic flux distribution in *Enterobacter aerogenes* mutants. *Int. J. Hydrogen Energy* 38, 13204–13215. doi:10.1016/j.ijhydene.2013.07.121
- Weiland, P., 2010. Biogas production: Current state and perspectives. *Appl. Microbiol. Biotechnol.* 85, 849–860. doi:10.1007/s00253-009-2246-7
- Wong, D.W.S., 2009. Structure and Action Mechanism of Lignolytic Enzymes. *Appl. Biochem. Biotechnol.* 157, 174–209. doi:10.1007/s12010-008-8279-z
- Wong, Y.M., Wu, T.Y., Juan, J.C., 2014. A review of sustainable hydrogen production using seed sludge via dark fermentation. *Renew. Sustain. Energy Rev.* 34, 471–482. doi:10.1016/j.rser.2014.03.008
- Woodward, J., Orr, M., Cordray, K., Greenbaum, E., 2000. Enzymatic production of biohydrogen. *Nature* 405, 1014–1015. doi:10.1038/35016633
- Wu, J., Ein-Mozaffari, F., Upreti, S., 2013a. Effect of ozone pretreatment on hydrogen production from barley straw. *Bioresour. Technol.* 144, 344–9. doi:10.1016/j.biortech.2013.07.001
- Wu, J., Upreti, S., Ein-Mozaffari, F., 2013b. Ozone pretreatment of wheat straw for enhanced biohydrogen production. *Int. J. Hydrogen Energy* 38, 10270–10276. doi:http://dx.doi.org/10.1016/j.ijhydene.2013.06.063
- Yan, B.H., Selvam, A., Xu, S.Y., Wong, J.W.C., 2014. A novel way to utilize hydrogen and carbon dioxide in acidogenic reactor through homoacetogenesis. *Bioresour. Technol.* 159, 249–257. doi:10.1016/j.biortech.2014.02.014

- Yoshida, T., Kojima, K., 2015. Toyota MIRAI Fuel Cell Vehicle and Progress Toward a Future Hydrogen Society. *Interface Mag.* 24, 45–49. doi:10.1149/2.F03152if
- Yu, J., Takahashi, P., 2007. Biophotolysis-based Hydrogen Production by Cyanobacteria and Green Microalgae. *Commun. Curr. Res. Educ. Top. Trends Appl. Microbiol.* 1, 79–89.
- Zhang, B., He, P.-J., Lü, F., Shao, L.-M., Wang, P., 2007. Extracellular enzyme activities during regulated hydrolysis of high-solid organic wastes. *Water Res.* 41, 4468–78. doi:10.1016/j.watres.2007.06.061
- Zhao, H., Lu, Y., Wang, L., Zhang, C., Yang, C., Xing, X., 2015. Disruption of lactate dehydrogenase and alcohol dehydrogenase for increased hydrogen production and its effect on metabolic flux in *Enterobacter aerogenes*. *Bioresour. Technol.* 194, 99–107. doi:10.1016/j.biortech.2015.06.149
- Zheng, H.S., Guo, W.Q., Yang, S.S., Feng, X.C., Du, J.S., Zhou, X.J., Chang, J.S., Ren, N.Q., 2014. Thermophilic hydrogen production from sludge pretreated by thermophilic bacteria: Analysis of the advantages of microbial community and metabolism. *Bioresour. Technol.* 172, 433–437. doi:10.1016/j.biortech.2014.09.020
- Zheng, Y., Zhao, J., Xu, F., Li, Y., 2014. Pretreatment of lignocellulosic biomass for enhanced biogas production. *Prog. Energy Combust. Sci.* 42, 35–53. doi:10.1016/j.pecs.2014.01.001
- Zong, W., Yu, R., Zhang, P., Fan, M., Zhou, Z., 2009. Efficient hydrogen gas production from cassava and food waste by a two-step process of dark fermentation and photo-fermentation. *Biomass and Bioenergy* 33, 1458–1463. doi:http://dx.doi.org/10.1016/j.biombioe.2009.06.008
- Zuroff, T.R., Curtis, W.R., 2012. Developing symbiotic consortia for lignocellulosic biofuel production. *Appl. Microbiol. Biotechnol.* 93, 1423–1435. doi:10.1007/s00253-011-3762-9

Annexes



Annex I: Result of rCCA obtained by confronted indexes calculated from sequences identified from inoculum to metabolites produced during dark fermentation



Annex II: Correlation found by rCCA by confronted indexes calculated from sequences identified in selected communities to metabolites produced during dark fermentation



PRODUCTION OF BIOLOGICAL HYDROGEN BY DARK FERMENTATION OF LIGNOCELLULOSIC RESIDUES: LINKS BETWEEN SUBSTRATE COMPOSITION AND STRUCTURE OF FERMENTATIVE MICROBIAL COMMUNITIES

Dark fermentation is a natural biological process that can be used to degrade organic waste, such as lignocellulosic components, and to produce energy under the form of hydrogen (H_2). The process is based on the activity of fermentative microorganisms in consortia that develop an efficient enzymatic activity to degrade complex substrate as lignocellulose. The presence of hydrogen non-producing or consuming bacteria in the system can result in gas production fluctuation. This thesis aims to highlight the mechanisms behind the selection of fermentative bacteria during dark fermentation, regarding the type of substrate and the final metabolisms. Batch experimentation brought out that both inoculum community structure and substrate composition, i.e. type of carbohydrate and degree of polymerisation, are important for the fermentation progress and thus hydrogen production. Inocula rich in bacteria from *Clostridium* sp. and *Enterobacter* sp. genera appeared to be more likely linked to H_2 production than mixed culture rich in microorganisms affiliated to *Bacillaceae* and *Paenibacillaceae* families. Results also showed hydrolytic bacteria from *Ruminococcaceae* and *Lachnospiraceae* families are preferentially selected on complex substrates. The use of fed-batch process on these types of substrates increased the experimental time and was used to enrich the microbial culture into these bacteria. Overall, this thesis provides new insights on the dynamics of bacterial communities within the dark fermentation process. It opens up new perspectives on mixed culture management to produced green and renewable hydrogen.

DEFENDED ON 18TH NOVEMBER 2016 AT:



WITH THE FINANCIAL SUPPORT OF :



INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE

Unité de recherche (UR0050) - Laboratoire de Biotechnologie de l'Environnement

102, avenue des Etangs
11100 Narbonne
France

Tél. : + 33 4 68 42 51 51
Courriel: lbe-contact@supagro.inra.fr
www.montpellier.inra.fr/narbonne