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Lien entre production de biohydrogène et métabolites microbiens par voie fermentaire et la composition des déchets organiques solides

PhD thesis 2012

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ALIMENTATION
AGRICULTURE
ENVIRONNEMENT

UNIVERSITE MONTPELLIER II
SCIENCES ET TECHNIQUES DU LANGUEDOC

T H E S E

pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE MONTPELLIER II

Discipline : Génie des Procédés

Ecole Doctorale : Sciences des Procédés – Sciences des Aliments

Devant être présentée et soutenue publiquement
par

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Le 20 juillet 2012

Titre :

Biohydrogen production and metabolic pathways in dark fermentation related to the composition of organic solid waste

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Abstract

This study aims to investigate the effect of solid substrates composition on hydrogen production performances, metabolic pathways and microbial community changes in batch reactor and their dynamics in continuous reactors (CSTR). Hydrogen is an ideal energy carrier which has gained scientific interest over the past decade. Biological H_2, so-called biohydrogen, can especially be produced by dark fermentation processes concomitantly with value-added molecules (*i.e.* metabolic end-products), while organic waste is treated. However, the effect of solid organic waste composition on biohydrogen production in dark fermentation has not yet been clearly elucidated.

In this study, a bibliographic review was made on hydrogen production from agricultural waste. This survey on literature showed that diverse performances were reported on hydrogen production due to the variability in substrate compositions and experimental conditions. After having optimized a protocol of biohydrogen potential test (BHP), a wide variety of organic solid substrates aiming to covering a large range of solid waste was tested to provide a comparable data analysis. The results of a PLS regression showed that only soluble carbohydrates or easily available carbohydrates correlated with hydrogen production. Furthermore, hydrogen yields correlated as well with butyrate H_2-producing pathway which is consistent with the literature knowledge. A predictive model of hydrogen yield according to carbohydrate content was proposed. Then, experiments were carried out in CSTR with Jerusalem artichoke tubers as a case study. It was shown that low organic loading rate favored continuous hydrogen production while higher organic loading introduced hydrogen competition pathways and decreased the overall hydrogen yields. Moereover, 16S rRNA gene based CE-SSCP profiles showed that increasing OLR had a significant effect on the microbial diversity by favoring the implementation of microorganisms not producing hydrogen, *i.e.* lactic acid bacteria

TITRE: Lien entre production de biohydrogène et métabolites microbiens par voie fermentaire et la composition des déchets organiques solides

Résumé

Cette étude vise à étudier l'effet de la composition de substrats organiques solides sur les performances de production d'hydrogène, les voies métaboliques associées et les changements des communautés microbiennes dans un réacteur discontinu (CSTR). L'hydrogène est un vecteur énergétique idéal qui a gagné en intérêt scientifique au cours de la dernière décennie. L'H_2 produit par voie biologique, ou biohydrogène, peut être produit par des procédés de fermentation sombre où les déchets organiques sont traités et avec la production de molécules à haute valeur ajoutée. Cependant, l'effet de la composition des déchets organiques solides sur la production de biohydrogène dans la fermentation sombre n'a pas encore été clairement élucidé.

Au cours de cette étude, une revue bibliographique a été réalisée sur la production d'hydrogène à partir de déchets agricoles. Cette revue montre qu'une large gamme de performances en hydrogène peut être observée principalement en raison de la variabilité dans les compositions en même type de substrats et des conditions expérimentales appliquées. Après avoir optimisé un protocole de test de potentiel biohydrogène (BHP), une grande variété de substrats organiques solides visant à couvrir un grand panel de déchets a été testée pour fournir des données comparables à analyser. Les résultats d'une régression PLS ont montré que seuls les sucres solubles ou facilement disponibles éteint corrélaient avec la production d'hydrogène. En outre, les rendements d'hydrogène corrélaient aussi bien avec l'accumulation de butyrate, principale voie productrice de bioH_2. Un modèle prédictif du rendement en hydrogène en fonction de la teneur en sucres a été proposé. Ensuite, des expériences ont été menées en réacteur continu (CSTR) avec le topinambour comme substrat solide. Il a été montré qu'une faible charge organique favorisait une production continue d'hydrogène tandis que l'accroissement de la charge organique introduisait la présence de voies concurrentes à la production d'hydrogène. De plus, les profils des empreintes moléculaires basées sur l'ADNr 16s ont montré que l'augmentation de la charge organique avait un impact significatif sur la diversité microbienne en favorisant l'implantation de microorganismes ne produisant pas d'hydrogène tels que des bactéries lactiques.

DISCIPLINE : Génie des Procédés, Biotechnologie de l'Environnement

MOTS-CLES : Biohydrogène, cultures mixtes, déchets organiques solides, voie fermentaire sombre

INTITULE ET ADRESSE DU LABORATOIRE : Laboratoire de Biotechnologie de l'Environnement. INRA, UR050, Avenue des Etangs, Narbonne F-11100, France

REMERCIEMENTS

Enfin, nous y sommes ! Une page vient de se tourner : l'aventure narbonnaise pendant 3 ans et demi. Quand je regarde derrière, je trouve que cette partie, après le temps passé parmi les gens du LBE, est la plus dure à rédiger car trop de mercis à dire et trop de souvenirs à conserver précieusement.

Tout d'abord, merci à Jean-Philippe Steyer, directeur de l'Unité, ainsi que co-directeur de thèse, pour sa bonne humeur et sa disponibilité. Je remercie également Jean-Philippe Delgenès, ancien directeur de l'Unité, pour m'avoir accueillie au sein du Laboratoire de Biotechnologie de l'Environnement (LBE). Merci pour ses conseils et son écoute. Mes plus sincères remerciements à Hélène Carrère (Directrice de Recherche à l'INRA de Narbonne), ma co-directrice de thèse, pour ses connaissances scientifiques, ses corrections et son implication tout au long de la thèse.

Je voudrais exprimer particulièrement ma profonde gratitude et l'immense reconnaissance envers Messieurs Eric Trably et Eric Latrille (deux ingénieurs de recherche à l'INRA de Narbonne), mes chers encadrants. Alors, pour leur soutien quotidien, pour leur encadrement, leurs conseils et leur amitié, surtout pour leur confiance et leur sincérité tout au long de la thèse, sans eux, je n'aurais pas pu m'épanouir professionnellement et scientifiquement aucours de ce travail de thèse. MERCI !

Je tiens à remercier l'allocation de recherche qui a soutenu financièrement cette thèse, également l'université Montpellier II et l'école doctorale des sciences des procédés et sciences des aliments.

Je tiens à exprimer ma gratitude à l'ensemble du jury, M. le professeur A. GRASMICK, de l'Université Montpellier II, pour avoir accepté la présidence de ce jury, et plus particulièrement les deux rapporteurs de ces travaux, Mme. M.-N. PONS, Directrice de Recherche du CNRS Nancy et M. le professeur A. PAUSS de l'Université Technologique de Compiègne. Je remercie aussi Mme M.

QUEMENEUR, Chargée de Recherche IRD de Marseille d'avoir accepté d'être l'examinateur de cette thèse. Merci ¨ vous tous d'avoir participé à l'évolution de cette thèse. Vos conseils, vos remarques et vos encouragements ont été bénéfiques.

J'adresse mes remerciements à l'ensemble du personnel du LBE. Tout d'abord mon grand remerciement aux chercheurs, Diana, Nicolas, Jean-Jacques, Jérôme et Nathalie, pour leurs enseignements, discussions intéressantes qui m'ont aiguillée sur mon orientation professionnelle. Je remercie ensuite tous les techniciens en particulier Anaïs, Valérie, Thierry, Guillaume et Philippe, pour leur sympathie et leur disponibilité. Merci à la <dream team>, Sylvie, Annie, Alexandra, Véronique, Isabelle et Nadine (ma voisine et collègue de covoiturage), pour leur bonne humeur permanente et leur aide dans les chemins sombres de l'administration. Merci aussi à Marjolaine pour ses conseils professionnels en rédaction scientifique.

Toutes ces années n'auraient pas été si gratifiantes et si riches sans des amitiés qui se sont confortées au fil du temps. J'exprime une pensée très chaleureuse à mes chers amis du bureau : Alexis, Juan, Julie, surtout les filles (mes coachs en français) Flo et Mélanie, elles m'accompagnent, m'encouragent, me corrigent…. Aussi Virginie, Vera avec qui j'ai fait de la piscine, le voyage de lavande et les soirées. J'ai pensé à tous les thésards et stagiaires avec qui j'ai passé des moments agréables (Maia, Mathieu(s) et sa femme Sophie, A2, Olivier…) et à qui je souhaite bon courage, notamment Charlie, Florian, Jean-Charles….

Enfin, et par-dessus tout, je souhaite adresser mes meilleurs sentiments à ma famille qui me soutient sans arrêt. Merci à mes parents qui me soutiennent à continuer mes études en France et m'ont permis de découvrir ce beau pays. Merci à mon amour « Hao » et mon petit cœur « Océanne », que dire que je les aime pour toujours….

RESUME ETENDU

L'hydrogène est l'élément le plus abondant dans l'univers. Il ne se trouve jamais sous sa forme élémentaire pure, mais est largement distribué dans les matières organiques sous une forme liée, de manière covalente, majoritairement avec les atomes de carbone, d'oxygène et d'azote. Sous sa forme gazeuse (H_2), l'hydrogène est considéré comme un vecteur énergétique idéal en raison de sa haute teneur en énergie par unité de masse (121 $kj.g^{-1}$), et au fait que sa combustion ne produise que l'eau. L'utilisation plus large de l'hydrogène comme vecteur énergétique à l'échelle mondiale aiderait à réduire la diffusion des émissions des gaz à effet de serre et limiterait alors l'influence négative sur leur changement climatique. Jusqu'à présent, l'hydrogène a été principalement utilisé dans les domaines de l'industrie tels que la fabrication de produits chimiques (ammoniaque), le raffinage des pétroles ou les filières alimentaires. Mais, récemment, un grand intérêt s'est porté sur la possibilité de développer des véhicules à hydrogène en remplacement des énergies fossiles. A titre d'exemple, la France est membre d'un partenariat international pour le développement de l'économie d'hydrogène, dont l'objectif est de fournir des véhicules à hydrogène compétitifs et à faibles coûts d'ici à 2020. En Février 2012, l'ADEME a d'ailleurs publié un appel à manifestation d'intérêt dédié au développement de tous types de véhicules équipés de piles à combustible fonctionnant à l'hydrogène.

Même si l'hydrogène ne pourra remplacer entièrement les combustibles fossiles ou non, il est attendu une demande toujours croissante en hydrogène dans le cadre du développement de technologies alternatives respectueuses de l'environnement notamment dans les transports. Les modes de production devront alors utiliser des ressources renouvelables de préférence. En effet, à l'heure actuelle, 96% de l'hydrogène est produit à partir de combustibles fossiles par reformage de gaz naturel, huiles lourdes

ou de charbon. Grâce au développement technologique considérable réalisé au cours de ces dix dernières années, la technique d'électrolyse de l'eau est maintenant plus intensément utilisée et a atteint près de 4% de la production mondiale en hydrogène ces dernières années. Cependant, ces techniques physico-chimiques ou electro-chimiques restent très coûteuses en énergie. Par conséquent, la production d'hydrogène par voie biologique, autrement appelé biohydrogène, qui nécessite une demande en énergie plus faible, a récemment attiré de plus en plus d'attention. En effet, le biohydrogène peut être produit directement à partir d'eau par biophotolyse à l'aide d'algues vertes; par biophotolyse indirecte à partir d'eau par des cyanobactéries; par photo-fermentation avec des bactéries photosynthétiques, et par voie fermentaire avec des bactéries anaérobies strictes ou facultatives. Parmi l'ensemble de ces possibilités, la voie la plus prometteuse paraît être la fermentation pour ses productivités élevées, la réutilisation de déchets organiques, la possibilité de réduire le volume total du procédé (procédé intensif), et l'adaptation des cultures mixtes microbiennes aux variations environnementales et fonctionnant en conditions non stériles. De plus, la possibilité d'adapter des installations existantes de digestion anaérobie offre l'avantage de permettre un développement rapide de la filière en cas d'émergence d'un marché biohydrogène économiquement viable.

Toutefois, la production de biohydrogène par voie fermentaire est encore loin du stade d'applications à l'échelle industrielle. Notamment, les limites à l'industrialisation des bioprocédés fermentaires de production d'hydrogène sont nombreuses : la première concerne le coût élevé du substrat. En effet, jusqu'à présent, les efforts de recherche ont principalement porté sur des substrats modèles tels que le glucose et le saccharose principalement issus de cultures énergétiques, filière qui ne peut être pas durable. Le biohydrogène pourrait être produit à moindre coût à partir de différents types de déchets comme les déchets solides agricoles ou organiques. Néanmoins, les connaissances accumulées sur les substrats simples ne peuvent pas être directement transférées à ces

déchets, les compositions et la structure étant beaucoup plus complexes. Une vision globale sur les facteurs structurels qui pourraient influer sur la conversion de ces substrats organiques en biohydrogène est actuellement manquante.

La seconde limitation concerne la stabilité de ces processus biologiques. En effet, les changements métaboliques et de populations microbiennes peuvent rapidement se produire en cultures mixtes, et notamment entre les processus de production d'hydrogène et les voies de consommation en hydrogène. Bien que, dans des conditions opératoires optimales de production d'hydrogène, les produits métaboliques finaux restent peu variés (acétate et butyrate), les changements d'alimentation en substrat ou les modifications en conditions opératoires conduisent à des modifications de populations microbiennes et voies métaboliques, parfois irréversibles et qui génèrent au grand panel de produits métaboliques sans production d'hydrogène. En conséquence, une connaissance approfondie de ces changements métaboliques et populationnelles en cultures mixtes est nécessaire afin de mieux comprendre les paramètres qui permettraient de contrôler et conserver le système fermentaire dans un état stable quel que soit le substrat organique utilisé.

La dernière limite concerne la structure de la communauté microbienne et les interactions microbiennes qui sont directement liées au réseau métabolique en cultures mixtes. En effet, l'hydrogène est l'un des principaux intermédiaires biochimiques dans les communautés microbiennes et est utilisé par de nombreuses espèces microbiennes comme vecteur énergétique pour transférer efficacement des électrons dans les milieux anaérobies. Ainsi, le lien entre structure des communautés microbiennes et métabolismes microbiens associés au processus de production d'hydrogène ne sont pas encore entièrement clarifiés. Encore peu de connaissance a été apportée dans la littérature sur les communautés microbiennes dans les procédés d'hydrogène fermentaires en lien avec

les dynamiques et les comportements microbiens.

Au cours de cette étude, une revue bibliographique a été réalisée sur la production d'hydrogène à partir de déchets agricoles et, plus généralement, issus des agro-industries. L'état des connaissances récentes sur la production de biohydrogène à partir de déchets agricoles par fermentation sombre a donc été présentée selon trois catégories de résidus: (i) les déchets générés directement à partir de pratiques agricoles (ii) les déjections et les effluents d'élevage et (iii) les déchets d'industries agro-alimentaires. Dans la littérature, il a été montré que les substrats organiques issus de ces trois catégories possèdent un potentiel intéressant pour la production d'hydrogène par fermentation, avec dans l'ordre décroissant: les déchets alimentaires, les résidus de récolte et les déchets d'élevage. Néanmoins, à ce jour des recherches permettant de mieux comprendre l'impact de la composition et de la nature du substrat sur les performances biohydrogène sont encore nécessaires pour en dégager de grandes tendances. Au cours de cette synthèse bibliographique il a été souligné que les procédés biologiques impliqués ne sont pas seulement influencés par la composition des déchets organiques, mais dépendent également des conditions opératoires appliquées: les principaux paramètres tels qu'un pH faible (environ 5,5), une faible pression partielle en hydrogène ($<10^{-3}$ atm), une température élevée (> 55 °C) ainsi que des communautés microbiennes acclimatées sont plutôt recommandés. Ces paramètres opératoires ne concernent pas seulement les rendements de conversion de la matière en biohydrogène, mais peuvent également influer sur le spectre en produits métaboliques et la structure des communautés microbiennes qui leur est associée. Un focus particulier a enfin été apporté sur les microorganismes composant les communautés microbiennes productrices d'hydrogène, et notamment il est important de distinguer trois catégories de micro-organismes dans ces cultures mixtes : le premier type concerne les bactéries productrices de biohydrogène. Les espèces microbiennes isolées en conditions mésophiles sont le plus fréquent

retrouvées au sein du genre *Clostridium*; néanmoins, en conditions thermophiles, des bactéries des genres *Thermoanaerobacterium* sp., *Caldicellulosiruptor* sp., *Clostridium* sp. et *Bacillus* sp. sont les plus fréquemment retrouvées dans les fermenteurs en cultures mixtes. La deuxième catégorie concerne les micro-organismes directement consommateurs d'hydrogène tels que les homoacétogènes, les sulfato-réductrices et les méthanogènes. Le dernier type concerne les micro-organismes «dissimulateurs du potentiel hydrogène» qui présentent des voies métaboliques concurrentes à la production d'hydrogène. La limitation de la présence des microorganismes consommateurs d'hydrogène et le contrôle des voies de consommation de l'hydrogène en cultures mixtes constituent les principaux défis pour améliorer la stabilité de bioréacteurs traitant des déchets agricoles. En conclusion, cette revue bibliographique a montré qu'une large gamme de performances en biohydrogène peut être observée à partir de substrats similaires principalement en raison de la variabilité dans les compositions des substrats et des conditions expérimentales appliquées.

L'objectif de la thèse est alors d'étudier l'influence de la composition des déchets organiques complexes, sur les performances et les dynamiques de production d'hydrogène et sur les voies métaboliques associées, en cultures mixtes.

Pour cela, nous avons réalisé un travail en deux parties:

Dans la première partie de l'étude, l'objectif était de déterminer quels composants des déchets organiques solides sont convertis en biohydrogène par des voies fermentaires, et donc d'en déterminer la relation existant entre la composition fine d'un substrat et les voies métaboliques fermentaires qui peuvent être associées. Pour ce faire, un protocole permettant de déterminer un «potentiel de production en biohydrogène (BHP)» a d'abord été optimisé afin de garantir des conditions opératoires strictement identiques entre les essais. Les résultats de la variation du type de tampon ainsi des

concentrations en substrat introduit ont montré que les conditions optimales pour la production d'hydrogène résultait d'un compromis entre la capacité à se maintenir dans une gamme de pH favorisant plutôt les bactéries productrices d'H_2, et limitant le développement des bactéries consommatrices d'H_2, notamment pour des pH supérieurs (illustré lorsque la concentration de tampon MES a été augmenté). Pour cette raison, une gamme optimale de la charge organique a été fixée entre 4 et 10 $g.L^{-1}$. Une trop forte charge organique peut conduire à l'inhibition de produits finaux en raison de la baisse de pH et un changement de voies métaboliques de production d'hydrogène vers des voies dissimulatrices ou de consommation en hydrogène.

Ensuite, l'impact de la composition en substrat sur les potentiels d'hydrogène a été évalué en utilisant le protocole préalablement optimisé. Une large gamme de déchets solides organiques (26) a alors été étudiée. Les compositions en sucres et en protéines de ces différents types de substrats ont été caractérisées: pour cela, une méthode d'hydrolyse à l'acide chlorhydrique 2 N a été modifiée et utilisée pour solubiliser en conditions douces les substrats testés, et ce afin d'éviter la formation de composés interférant sur la quantification des sucres libérés (furfural, HMF). Alors, les concentrations en sucres solubilisés ont été déterminées. De la même manière que pour la détermination de la teneur en sucres dans les déchets solides, les compositions en protéines ont été déterminées indirectement après solubilisation (solution d'hydroxyde de sodium 0.5M), et analyse par une méthode colorimétrique (acide bicinchoninique). Un test statistique d'ANOVA sur les rendements d'hydrogène à partir de ces substrats organiques solides a montré que les compositions des substrats significativement influencent sur les potentiels hydrogène et les substrats riches en sucres ont les potentiels hydrogène le plus élevés. En revanche, les rendements en hydrogène les plus faibles ont été observés pour des substrats riches en protéines, indiquant que la teneur en protéines était facteur défavorable pour la production de biohydrogène. Une analyse

statistique multilinéaire a été utilisée et a permis de montrer ces effets de la composition des substrats sur la production d'hydrogène, et sur les distributions des métabolites. En outre, la régression PLS a souligné que la concentration en sucres hydrolysables par HCl 2N et l'accumulation du butyrate étaient fortement corrélés avec les rendements en hydrogène. Un second modèle reliant la production en biohydrogène à la concentration en sucre hydrolysables par HCl 2N (nommée $sucre_{HCl)}$ a alors été construit. Ce modèle prédictif permet d'estimer approximativement le rendement en hydrogène (BHP) en fonction des concentrations en sucres hydrolysables.

Une caractérisation plus poussée sur dix substrats lignocellulosiques sélectionnés a été réalisée en utilisant une méthode de fractionnement de la matière, la méthode Van Soest, où les matières organiques sont scindées en plusieurs fractions: solubles dans un détergent neutre (fraction NDF), hémicelluloses, celluloses et lignines. Ensuite, une analyse par régression non linéaire (PLS) a montré que seulement la partie soluble des substrats lignocellulosiques était corrélée positivement avec les rendements en hydrogène. Une relation linéaire a été trouvée entre la concentration en sucre dans la fraction NDF (nommée $sucre_{NDF)}$ et les rendements hydrogènes. En conclusion, les deux méthodes utilisées pour estimer la concentration en sucres à partir de substrats lignocellulosiques a illustré que seulement les sucres facilement hydrolysables étaient bio-disponibles pour leur dégradation via les voies de production d'hydrogène, c'est à dire principalement les voies acétate et butyrate. Nos résultats montrent qu'il est possible de prédire des rendements de production d'hydrogène par la simple analyse de la composition des déchets organiques, en particulier en $sucres_{HCl}$. Le niveau de butyrate dans le milieu paraît alors être un bon indicateur d'un processus fonctionnel d'un métabolisme orienté vers la production d'hydrogène.

La deuxième partie de l'étude a visé à étudier les performances et dynamiques de

production en hydrogène dans des réacteurs fonctionnant en mode continu, à partir d'un substrat modèle. Pour cela, le topinambour a été choisi pour ses teneurs élevées en sucres, notamment l'inuline, un polymère de fructose, et pour son potentiel hydrogène élevé. Dans cette partie, il s'agissait de voir si l'utilisation des substrats dans le réacteur continu est la même que dans le batch, c'est-à-dire que si les sucres facilement hydrolysables dans substrats complexes sont bio-accessible via la production d'hydrogène. Puis, la charge organique, facteur très important pour les performances de production d'hydrogène, qui devrait été opérée dans une gamme optimale comme été montrée précédemment dans le batch. L'effet de la charge sur les rendements en hydrogène a donc été étudié avec des racines de topinambour, et l'impact sur les changements des communautés microbiennes a ainsi été évalué. Les résultats ont montré la possibilité d'utiliser du topinambour en suspension pour produire de l'hydrogène par des microorganismes indigènes par fermentation sombre et en fonctionnement continu, en duplicats (réacteurs A et B). Dans le réacteur B, un rendement en hydrogène de 1,5 ± 0,3 mol_{H2}.mol $sucre_{HCl}^{-1}$ a été obtenu à faible charge de 22 ± 1 g $sucre_{HCl}$. $L^{-1}.d^{-1}$, en comparaison avec 1,0 ± 0,1 mol_{H2}.mol $sucre_{HCl}^{-1}$ à une forte charge de 45 ± 3 g $sucre_{HCl}$. $L^{-1}.d^{-1}$. Cependant, dans le réacteur A, la charge organique n'a pas montré d'effet significatif sur les rendements d'hydrogène (soit 1,4 ± 0,2 mol_{H2}.mol $sucre_{HCl}^{-1}$ et 1,3 ± 0,2 mol_{H2}.mol $sucre_{HCl}^{-1}$ pour les deux charges dans le réacteur A). Afin d'expliquer ces résultats, les voies métaboliques ont été étudiées, et il a été observé que le butyrate et l'acétate étaient les principaux produits métaboliques à faible charge, et que le lactate et le caproate sont devenus des produits métaboliques co-dominants à forte charge, ce qui suggère l'installation de voies dissimilatrices (lactate) et consommatrices d'H_2 (caproate) à une charge élevée. De plus, une régression PLS sur ces résultats a montré une corrélation forte entre le butyrate et le rendement en hydrogène, comme montré précédemment en batch (BHP): parmi l'ensemble des produits métaboliques, la concentration de butyrate représente un excellent indicateur de voies prédominantes de

production en hydrogène. Afin de mieux comprendre les phénomènes liés aux changements de métabolismes à forte charge, et déterminer ainsi s'il s'agissait de changements métaboliques ou populationnels, la structure des communautés microbiennes a été étudiée par typage moléculaire sur le gène codant pour l'ARNr 16S par une technique de CE-SSCP. Il a été observé que l'augmentation de la charge avait eu un effet significatif sur la diversité microbienne en favorisant la mise en œuvre d'autres micro-organismes, avec comme hypothèse la présence de bactéries lactiques.

L'ensemble des résultats obtenus au cours de cette thèse ont fourni des perspectives intéressantes en deux points: tout d'abord en ce qui concerne le génie des procédés mais aussi dans le domaine des communautés microbiennes. En effet, dans cette étude, une relation entre sucres facilement hydrolysables et production d'hydrogène a été montrée. En termes de génie des procédés, le prétraitement du substrat afin d'améliorer la solubilisation des sucres constitue donc une étape essentielle à étudier afin de valoriser les déchets en termes d'hydrogène et d'acides gras volatils par fermentation sombre, et plus particulièrement dans le cas de substrats lignocellulosiques. Toutefois, le prétraitement doit aussi tenir soigneusement compte des types de sucres constituant les substrats. A titre d'exemple, le topinambour contient une grande quantité d'inuline, un polymère du fructose, qui produit, après l'hydrolyse avec un acide fortement concentré, de nombreux HMF (5-hydroxy-2-méthylfurfural), lui-même étant un inhibiteur fort de la production d'hydrogène. Ainsi, il serait intéressant d'étudier des prétraitements doux sur topinambour pour améliorer la solubilisation d'inuline et d'éviter la production d'inhibiteurs. Egalement, l'utilisation d'enzymes biologiques naturellement présentes dans les micro-organismes peut s'avérer être un bon choix. Comme les polymères de l'inuline sont intégrés dans une matrice lignocellulosique, une combinaison de prétraitements d'hydrolyse par des enzymes issues de champignons (capables d'hydrolyser la lignocellulose) et la fermentation sombre avec des espèces cibles, telles

que *Clostridium thermocellum* en cultures mixtes pourrait être proposée.

De plus, dans les expériences en réacteurs continus (CSTR), environ 60% des solides totaux de racines du topinambour ont été convertis en hydrogène et métabolites. Les substrats restants et l'énergie incluse dans les métabolites produits doivent être valorisés. Une étude intéressante pourrait alors envisager d'améliorer le rendement total de conversion de la matière. Par exemple, la combinaison de la fermentation en voie sombre avec un procédé de photofermentation ou de méthanisation pourrait être intéressante. Enfin, une fois l'établissement d'une procédure en deux (voire trois) étapes stable, des expériences à l'échelle pilote pourraient être testées ainsi qu'une analyse de cycle de vie afin d'estimer au mieux les impacts globaux de ces procédés. Ce sont les pré-étapes essentielles avant l'industrialisation des procédés de production de biohydrogène.

De plus, la dynamique des populations microbienne a prouvé que, à faible charge, seules les bactéries qui produisent de l'hydrogène (par exemple, *Clostridium* spp.) sont prédominantes et montrent de meilleures performances en hydrogène. Ceci suggère donc de contrôler la communauté microbienne en opérant une charge appropriée. Des travaux de recherche devraient ainsi approfondir la connaissance sur les mécanismes de réduction de la diversité microbienne par ses paramètres opératoires. Par exemple, les paramètres opératoires tels que la concentration du substrat, et temps de séjours hydraulique pourraient être modifiés tout en gardant la même charge afin d'étudier l'impact de la nature du substrat sur cette diversité microbienne. Analyser la communauté microbienne au cours de ces conditions en ciblant également spécifiquement la diversité fonctionnelle d'un gène codant pour une hydrogénase *hyd*A ([Fe-Fe] hydrogénases), permettrait alors de comparer la fonction, la dynamique et la sensibilité des souches productrices d'hydrogène par rapport à la diversité microbienne

totale.

Pour conclure, cette thèse a contribué à améliorer la compréhension de l'impact de la composition des déchets solides organiques sur la production d'hydrogène en proposant une généralisation des résultats, et en y incluant une analyse des voix métabolique impliquées. Ceci permettra de prédire et concevoir un meilleur processus de valorisation des résidus agricoles et mieux suivre les performances des procédés en impliquant le butyrate comme un indicateur des voies de production en biohydrogène

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ABBREVIATIONS

ASBR: Anaerobic Sequencing Batch Reactor

ATP: Adenosine triphosphate

sCSTR: Semi-Continuous Stirred Tank Reactor

CSTR: Continuous Stirred Tank Reactor

COD: Chemical Oxygen Demand

FISH: Fluorescent *In Situ* Hybridization

HRT: Hydraulic Retention Time

HAB: HomoAcetogenic Bacteria

LAB: Lactic Acid Bacteria

LCFA: Long Chain Fatty Acids

MPB: Methane-Producing Bacteria

MFC: Microbial Fuel Cells

$N\text{-}NH_4$: Nitrogen in ammonia form

NADH: Reduced nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

OLR: Organic Loading Rate

PCR-CE-SSCP:Polymerase Chain Reaction-Capillary electrophoresis-single strand conformation polymorphism

SRB: Sulfate-Reducing Bacteria

TS: Total Solids

UASB: Upflow Anaerobic Sludge Blanket

VS: Volatile solids

VFAs: Volatile Fatty Acids

All the measurements of produced biogas volume in this thesis had been performed at 25 °C (room temperature) and 1 atm. The factor to calculate to standard condition (0 °C, 1 atm) is 0.9161.

GENERAL INTRODUCTION

Hydrogen is the most abundant element in the universe. Hydrogen element is never found in its pure elemental form but is widely distributed in organic matter through covalent bounds with, mostly, carbon, oxygen and nitrogen atoms. In its gaseous form (H_2), hydrogen is one of the main biochemical intermediates in microbial communities and is utilized by many microbial species as energy carrier to efficiently transfer electrons in anaerobic environments. Thus, similarly, hydrogen has recently been considered as an ideal energy carrier by humans due to its high energy content per weight (121 $kj.g^{-1}$) and to the fact that its combustion only produces water. Using hydrogen as a global and clean energy carrier would also help to reduce greenhouse gas emissions and limit further negative influence on climate change. Up to now, hydrogen has been mainly used in industry fields such as chemical manufacturing, petroleum refining or metal fabrication. But a great interest has lately increased on the possibility of developing hydrogen-powered vehicles. As an example, France is a member of an international partnership for hydrogen economy having the objective to provide competitive low cost hydrogen cars by 2020. In February 2012, the ADEME published a call for projects dedicated to all types of vehicles equipped with hydrogen fuel cells.

No matter whether hydrogen will replace fossil fuels or not, the increasing demand of hydrogen in the framework of the development of technologies using environmental friendly energy carriers must lead to production modes using preferentially renewable resources. At present, hydrogen is commercially produced at 96 % from fossil fuels (natural gas, heavy oils or coal). Beneficiating of an extensive development in the past ten years, water electrolysis is now more intensively utilized and has reached up to 4 % of the total hydrogen production the last years. However, all of these techniques are high energy-consuming. Therefore, the hydrogen production by a biological route (biohydrogen), which requires lower energy demand, has gained more and more attention recently. Biohydrogen can indeed be produced by direct water bio-photolysis by green algae, indirect water biophotolysis by cyanobacteria,

photo-fermentation by photosynthetic bacteria, and dark-fermentation by strict and facultative anaerobic bacteria. The most promising ways is the dark-fermentation for its high productivity rate, the reuse of organic waste and, concomitantly, the possibility of reducing the total volume of the process and the high adaptation of microbial mixed cultures that does not require sterile conditions to be operated. Moreover, the possibility of using existing facilities of anaerobic digestion provides technical advantages to make rapidly the biohydrogen market economically viable.

However, biohydrogen production is still far from industrial-scale applications. The main limitations to the industrialization of bioprocesses of hydrogen production by dark fermentation are numerous. The first issue concerns the high environmental and economic costs of the substrate. Indeed, until now, the research efforts have principally focused on model substrates such as glucose and sucrose mainly produced by energetic cultures which is not sustainable. Biohydrogen could be produced at lower environmental and economic costs from many different kinds of waste such as agricultural or organic solid waste. Nevertheless, the knowledge accumulated on glucose or sucrose cannot be directly transferred to these waste as their composition and structure are much more complex. Moreover, a global overview on the structural factors impacting the conversion of raw waste substrates to hydrogen is presently rather limited.

The second limitation to industrialization is the stability of such biological processes. Indeed, metabolic shifts could rapidly occur between hydrogen-producing and hydrogen-consuming microbial pathways in pure and mixed culture fermentation processes. Although, under optimal operating conditions, few major end-products (*e.g.* acetate and butyrate) are generated concomitantly with hydrogen, changes in substrate feeding or slight changes in operating conditions could lead to shifts in microbial populations and metabolic pathways leading to the production of a broad spectrum of end-products. As a consequence, a deeper knowledge on these metabolic changes in mixed cultures is recommended to keep such fermentative systems in steady state whatever the organic substrate.

The last limitation concerns the microbial community, which is directly related to the metabolic network in mixed cultures. In fact, microbial community structures and microbial metabolic relationships in continuous fermentative hydrogen process have not been yet clarified. Only little is known about fermentative hydrogen microbial communities and on microbial dynamics and behaviors in hydrogen-producing bioprocesses.

The present work aims to investigate the influence of complex organic waste composition on hydrogen performances as well as on mixed culture metabolic pathways and microbial dynamics.

In the first part of the study, the objective was to determine which components of the organic solid waste are converted to biohydrogen, and the relationship between the global composition of the substrate and the metabolic pathways. For this, a protocol for determining the "biohydrogen potential" was firstly optimized in batch fermenters. A large range of organic solid waste was then investigated with this "optimized" protocol to produce biohydrogen by dark fermentation. Meanwhile, the compositions of these different types of substrates were characterized. The statistical multilinear analysis was used to show the effect of substrate compositions to hydrogen yields and metabolite distributions, and a predictive model of biohydrogen yield according to the substrate composition was built.

The second part of the study aims to investigate the hydrogen production performances and dynamics in continuous stirred tank reactor systems. For this, the Jerusalem artichoke plant was selected as a case study of solid substrate for its high contents in carbohydrates, *i.e.* inulin. The effect of organic loading rate on hydrogen yields was studied with Jerusalem artichoke bulbs, and the impact on microbial community changes was evaluated.

This thesis is organized in four chapters, as follows:

The first chapter corresponds to a literature review on biological hydrogen production from agri-cultural waste by dark fermentation. It consists of a state of the art on hydrogen as

energy carrier, on the biological hydrogen potentials of organic agricultural wastes; on reactor operation parameters for further optimization of hydrogen production; on microbial communities found in fermentative H_2-producing conditions.

The second chapter describes all material and methods utilized in the experiments as well as the statistical analysis.

The third chapter "Results and Discussion" represents the results obtained, divided into two sub-chapters. The first subchapter concerns the optimization of a protocol to evaluate the biohydrogen potentials in batch tests. Then a wide range of organic substrates was used to evaluate the influence of the substrate composition on hydrogen production. Statistical analysis of the results using PLS regression was then performed to build a predictive model of their biohydrogen potential. The second part of the results is composed of experiments from raw materials in continuous stirred tank reactors (CSTR). The effect of organic loading rate on microbial community dynamics was investigated from a raw substrate model: the Jerusalem artichoke plant.

Finally the last chapter includes an overall discussion of the observations and proposes several perspectives for further research work.

The results obtained in this work have already been published, submitted or are in preparation in journals, as shown below:

1. GUO XM, TRABLY E, LATRILLE E, CARRERE H, STEYER JP. Hydrogen production from agricultural waste by dark fermentation: A review. *International Journal of Hydrogen Energy* 2010; 35 (19): 10660-10673.
2. GUO XM, LATRILLE E, TRABLY E, CARRERE H, STEYER JP. Substrate mapping by PLS regression analysis and prediction of biological hydrogen potential tests from solid organic waste. *Waste management.* (submitted)

3. GUO XM, TRABLY E, LATRILLE E, CARRERE H, STEYER JP. Effect of organic loading rate on biohydrogen production from Jerusalem artichoke. (in preparation)

The results obtained in this work have also been presented in national and international conferences

-oral communications:

1. Guo XM, Trably E, Latrille E, Carrere H, Steyer JP. Caractérisation de plusieurs déchets agricoles et détermination de leur potentiel de production de biohydrogène par voie fermentaire. Journées GDR $BioH_2$ – Voies biologiques et biomimétiques de synthèse et d'utilisation de l'hydrogène. 5 au 8 Octobre 2009, La Londe les Maures, France

2. Guo XM, Latrille E, Trably E, Carrere H, Steyer JP. Correlation analysis with PLS regression of biological hydrogen potential (BHP) tests of solid organic waste. Thirteenth International Waste Management and Lanfill Symposium – International Waste Working Group –IWWG). October 3rd – 7th 2011, Cagliari, Italy

-poster:

1. Lesteur M, Guo XM, Latrille E, Trably E, Roger JM, Bellon-Maurel V, Gonzalez C, Junqua G, Steyer JP. Fast prediction of the hydrogen potential of solid waste by Near Infrared Spectroscopy. 12th World Congress on Anaerobic Digestion. October 31st – November 4th 2010, Guadalajara, Mexico

CHAPTER I. LITERATURE REVIEW

This chapter of literature review was adapted from GUO XM, TRABLY E, LATRILLE E, CARRERE H, STEYER JP. Hydrogen production from agricultural waste by dark fermentation: A review. International Journal of Hydrogen Energy 2010; 35 (19): 10660-10673. DOI: 10.1016/j.ijhydene.2010.03.008

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In this chapter, a comprehensive literature review on hydrogen production and applications is proposed. A special focus is made on biohydrogen production by dark fermentation from solid waste. First, the up-to-date available data dealing with the potential in hydrogen production from diverse types of solid waste has been compiled. Three distinct potential and readily available types of feedstock were distinguished: (1) the crop residues or so-called lignocellulosic substrates where enhanced hydrogen yields are obtained only when pre-treatments are applied; (2) livestock waste exhibiting inhibitory effects on hydrogen yields; and (3) food waste where process conditions are strongly influencing the conversion of organic matter to biohydrogen. Second, the operating parameters influencing the H2-producing biological processes such as pH, hydrogen partial pressure, temperature, HRT and OLR, are discussed. Third, an overview on the potential application of biohydrogen processes in a concept of biorefinery is presented. The combination of bioH2 production to additional bioprocesses such as methanogenesis in anaerobic digestion, photo-fermentation and microbial electrochemical cells are also considered to improve the total energy recovered in such streams of waste treatment. Finally, fundamentals in microbiology are presented with a special attention on the microbial specificities of mixed culture involved in metabolic pathways where hydrogen is a key element of the overall process, either as a product (H_2 generation) or as substrate (H_2 consumption).

I.1 Overview on hydrogen production from solid waste

I.1.1 Hydrogen as an ideal energy carrier

Hydrogen is the simplest, lightest and most abundant chemical element found in Nature. However, hydrogen is never found in its pure elemental form but is widely distributed in organic matter through covalent bounds with, mostly, carbon, oxygen and nitrogen atoms. Only the diatomic hydrogen gas, so-called H_2, corresponds to the pure form of hydrogen but is rarely found in Nature, with abundance lower than 1 ppm in the atmosphere. Hydrogen gas

was first observed in the early 16th century by Henry Cavendish, a British scientist, who steeped metals in strong acids. This strange highly inflammable gas which forms water after burning was later named "hydro-gen gas" as a atin equivalence for "water-forming gas". Later, in the novel "The mysterious island" published in 1874, Jules Verne imagined the use of hydrogen as a substituted energy source. But this is not actually a well-adapted definition: hydrogen is not an energy source since hydrogen has to be produced from other primerary energy sources such as fossil fuels, but it can be considered as an ideal energy carrier (Vander-Poorten and Crappe, 2005). Indeed, and according to the academic definition of an energy carrier as previously established in the international legislation ISO 13600, an energy carrier is "a substance or a phenomenon which can be used to produce mechanical work or heat or to operate chemical or physical processes". The main current energy carriers are solids (coal), liquids (gasoline diesel oil, jet fuel, ethanol, methanol, liquefied gases, fatty esters), gaseous (natural gas, synthetic gas) and electricity (Mazza and Hammerschlag, 2004). Hydrogen, as energy carrier, presents several advantages related to its physico-chemical properties: first, at standard temperature and pressure (0 °C, 1atm), hydrogen gas is odourless, colourless, non-metallic, tasteless, non-toxic, highly combustible, and has the highest energy-to-weight ratio of 121 $MJ.kg^{-1}$. In other words, 1 kg of hydrogen represents the same amount of energy as 2.1 kg of natural gas or 2.8 kg of gasoline. Second, the combustion of hydrogen gas produces only water and, therefore no greenhouse gases are directly generated during the burning process. Moreover, the secondary combustion pollutants such as NOx are significantly reduced. Finally, the hydrogen combustion presents higher flame temperatures than other gases, with 2318 °C and 3000 °C in air containing 29 % H_2 in volume and in pure oxygen atmosphere, respectively. By comparison, the highest temperature that can be reached when methane is burned in air is only about 2000 °C, much less than any values reported from hydrogen combustion (Haemers G *et al*., 2006).

Presently, hydrogen has not been commercially used as energy carrier, but is only utilized in petro-chemical industries. Today, the commercial production of hydrogen has reached

more than 50 million tons every year (CEA, 2007). H_2 is industrially used for the production of ammonia (at 34 %), for petroleum desulfurization and methanol production in refineries (at 51 %), and in the sectors of metallurgy, electronics, pharmacology, food processing and for space usage (at 15 %) (CEA, 2007). From an engineering point view, hydrogen is produced from three main sources: fossil fuels, nuclear plants and renewable sources (Figure I-1). To date, 96 % of hydrogen is produced from fossil energy (Nath and Das, 2003). More precisely, steam reforming of natural gas by hot water is the most common process having the lowest cost of production if compared to other ways of hydrogen production. But thermal-chemical processes of natural gas steam reforming present several drawbacks and, notably, a massive reject of CO_2 into the atmosphere which leads to worsen the greenhouse effect. Since fossil fuel supplies are declining, the investigation on other modes of production of energy has become very essential and of political, sociological and scientific interests. An alternative way is to separate the oxygen atoms and hydrogen molecules from water by electrolysis. The promising point of this process is its no-greenhouse gas emission. Beneficiating of an extensive development in the past ten years, water electrolysis has been more widely utilized and represents now up to 4 % of the total hydrogen produced in the past years. Another method is to decompose water by thermochemical cycles associated to low electrical currents. This method needs for dissociating the hydrogen molecule, temperatures between 800 °C to 1000 °C. Such temperatures and electrical currents are obtained in nuclear reactors of new generation and in new solar power plants. The final possibility is to convert the renewable energy to hydrogen through thermal gasification of biomass or, by biological ways either with microalgae for biophotolysis of water, or if the organic matter is biologically degradable, by using fermentative hydrogen-producing bacteria (CEA, 2007). Producing hydrogen by biological ways shows several advantages since it can be operated independently from fossil fuel availability, the net greenhouse gas emission is zero since carbon is biologically recycled, and it requires less energy than thermal-chemical processes. However, biological processes are limited by the microbial metabolism and thermodynamics, leading to low hydrogen yields and productivities.

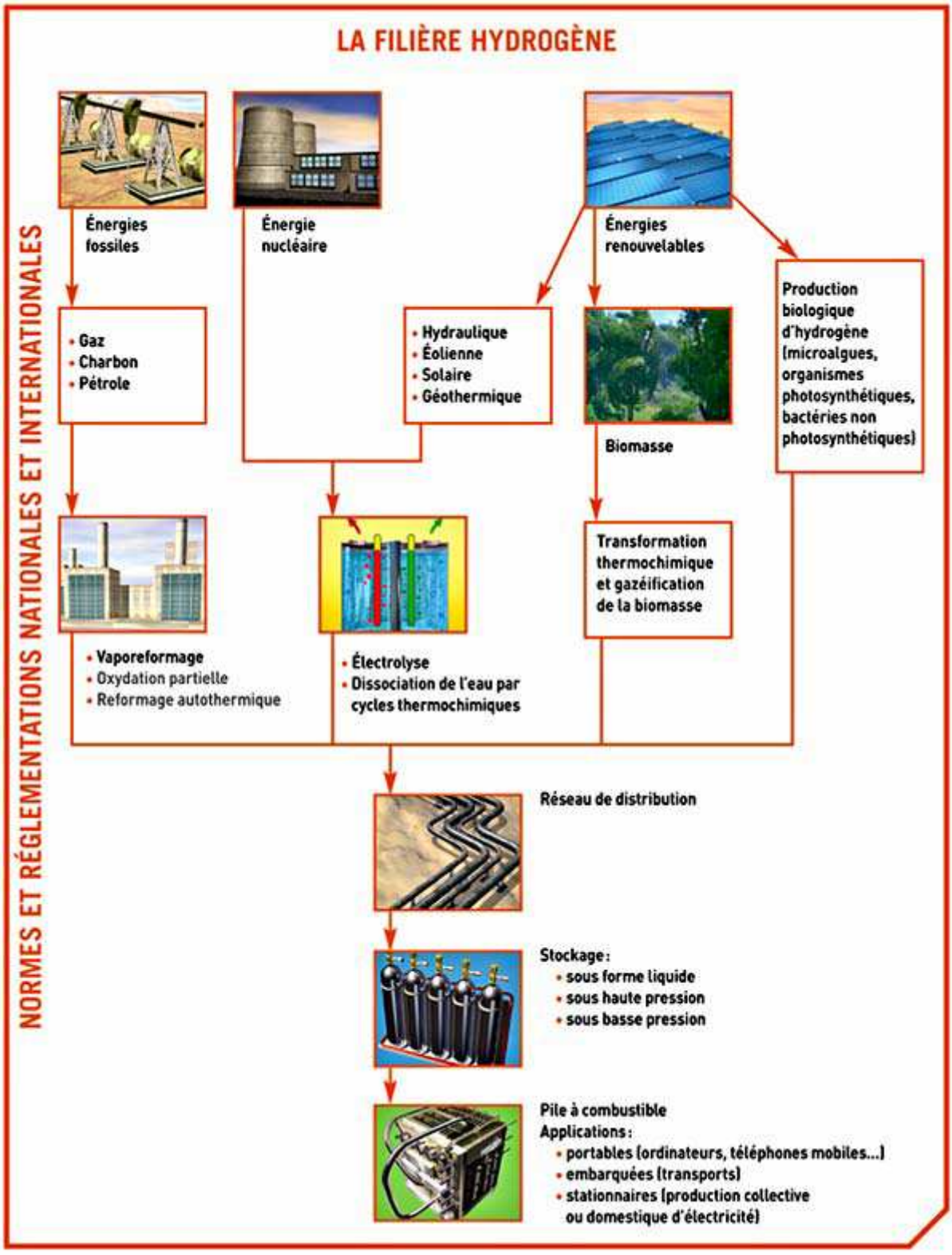


Figure I-1: Hydrogen generation: from energy source to application (CEA, 2007)

I.1.2 Hydrogen production by biological ways, so-called biohydrogen

One promising alternative way to produce hydrogen, without any dependency on fossil fuels and potentially at a low cost, is based on biological technologies. Hydrogen produced by microorganisms from renewable sources such as biomass, water or organic waste is called "biohydrogen". The detailed bioprocesses to produce hydrogen are described below, including direct water biophotolysis by green algae, indirect water biophotolysis by cyanobacteria, photo-fermentation by photosynthetic bacteria and dark fermentation by strict or facultative anaerobic bacteria.

I.1.2.1 Direct biophotolysis processes

In direct biophotolysis process, the green algae capture light energy to convert directly water to hydrogen and oxygen (Hallenbeck and Benemann, 2002). As showed in Figure I-2, the light energy absorbed by the photosystems generates electrons which are transferred to ferredoxin. From the reduced ferredoxin, electrons are then accepted to generate hydrogen catalysed by a reversible hydrogenase enzyme. The attractive point of this biological process is the use of low cost and very abundant sources, such as solar energy and water. However, the oxygen sensitivity of the hydrogenases remains a key problem since the hydrogenase activity is irreversibly inhibited in presence of oxygen. Moreover, the energy conversion efficiency and productivity of such processes are very low. As maximal yields, a solar conversion efficiency of 10 % was showed in a laboratory experiment with the green microalgae *Chlamydomonas reinhardtii*, under low-light intensities and very low partial pressures of oxygen (Greenbaum, 1988). Otherwise, in normal algae culture conditions, the reported conversion efficiencies remain mainly below 1 % (Hallenbeck and Benemann, 2002). Furthermore, the average rates of hydrogen production reported in the literature for *Chlamydomonas reinhardtii* are ranging from 0.07 $mmol_{H2}.L^{-1}.h^{-1}$ to 0.089 $mmol_{H2}.L^{-1}.h^{-1}$ (Melis *et al.*, 2000; Kosourov *et al.*, 2002).

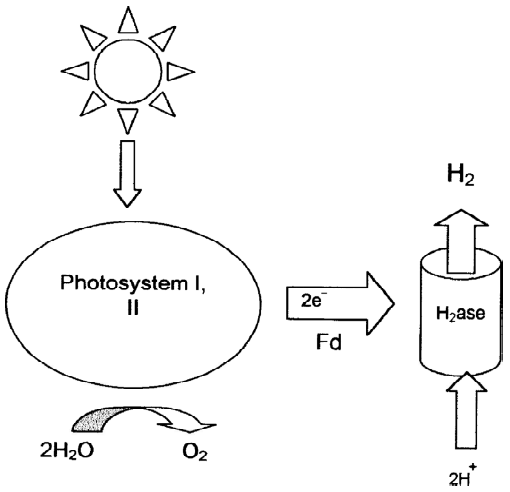


Figure I-2: Principles of direct biophotolysis operated by microalgae (from Hallenbeck and Benemann, 2002)

I.1.2.2 **Indirect biophotolysis process**

The overall principle of indirect biophotolysis process is similar to that of direct biophotolysis: to split water into oxygen and hydrogen through solar energy conversion. The difference is that in indirect biophotolysis, the oxygen and hydrogen generation biological systems are physically separated in two distinct compartments to avoid the oxygen inhibition of enzymes involved in hydrogen production, *i.e*. the nitrogenases (Hallenbeck and Benemann, 2002). Figure I-3 presents an example of indirect photolysis in nitrogen-fixing cells, so-called heterocysts, by cyanobacteria. In the left cell, the oxygen evolution is carried out through photosynthesis. The solar energy is converted to carbohydrates, here represented as "CH_2O", which are energy-rich sugar polymers used as electron sinks. In a second stage, the nitrogen-fixing heterocysts provide an oxygen-free environment where nitrogen is reduced to ammonia (NH_4^+) with the help of nitrogenases (labelled as Nase in Figure I-3). Under nitrogen starvation, protons in excess are transferred to hydrogen gas by nitrogenases using high amounts of cellular energy in forms of ATP, - 16 moles ATP per mole of hydrogen, generated from the energy-rich carbohydrates. Carbohydrates are indeed consumed at a low light intensity and with a metabolic stress (often S or N limitations) (Yu and Takahashi, 2007). This biological process takes advantages of the direct production of hydrogen directly from

water, and its ability of fixing N_2. However, the reported performances of hydrogen production are in a very wide range because of the wide variety of cyanobacteria capable of indirect biophotolysis. Indeed, cyanobacteria are belonging to a large group of photoautotrophic microorganisms, including blue-green algae, cyanophyceae, and cyanophytes. Nonetheless, the H_2 productivity in indirect biophotolysis is rather low and the solar energy conversion efficiency by nitrogenase remains always lower than 1 % (Yu and Takahashi, 2007). Interestingly, higher rates of hydrogen production have been reported in *Anabaena* sp., and the highest specific rate is of 12.6 $mol_{H2}.\mu g_{proteins}^{-1}.h^{-1}$, equivalent to 0.355 $mmol_{H2}.L^{-1}.h^{-1}$, reported from *Anabaena variabilis* cultivation (Levin *et al.*, 2004).

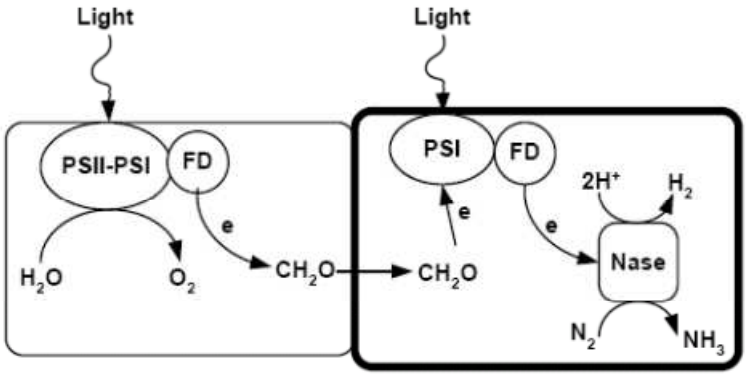


Figure I-3: Principles of indirect biophotolysis: example of viable cells and nitrogen-fixing heterocysts partitioning in cyanobacteria generating biohydrogen with the help of a nitrogenase. The right cell corresponds to the no-oxygen heterocyst cell where hydrogen is generated in absence of N_2 (from Yu and Takahashi, 2007).

I.1.2.3 **Photofermentative processes**

Photo-fermentation is a biological process that catalyses protons reduction to hydrogen by a nitrogenase under nitrogen-starvation conditions and through light energy concomitantly with the reduction of organic compounds (Figure I-4). The organic compounds that can be used, do not only consist in model carbohydrate sources such as glucose or sucrose, but also can be extended to simple organic waste or end-products issued from fermentative

bioprocesses, such as Volatile Fatty Acids as presented in the following paragraph (Fang *et al.*, 2006; Jeong *et al.*, 2008; Keskin *et al.*, 2011).

Basak and Das (2007) reported in a perspective review about purple non-sulfur photosynthetic bacteria usage, that the most widely used photosynthetic bacteria for photo-fermentative hydrogen production are *Rhodobacteri sphaeroides* O.U001, *Rhodobacter capsulatus*, *R.sphaeroides-RV, Rhodobacter sulfidophilus. Rhodopseudomonas palustris* and *Rhodospirillum rubrum* (Basak and Das, 2007). Hydrogen-producing photo-fermentation process has been extensively studied over the past ten years. Most of the research works have been carried out with pure cultures in batch tests. The average hydrogen yield and production rate of *R.sphaeroides* from glucose were estimated at 4.91$mmol_{H2}.g_{glucose}^{-1}$ and 3.59 $mmol_{H2}.L^{-1}.h^{-1}$, respectively (Fang *et al.* 2006). Another study on starch fermented by *Rubrivivax gelatinosus* showed that the hydrogen yield and rate reached 6.12 $mmol_{H2}.g_{glucose}^{-1}$ and 0.54 $mmol_{H2}.L^{-1}.h^{-1}$ (Bianchi *et al.*, 2010). The highest value of hydrogen productivity, *i.e.* 5.27 $mmol_{H2}.L^{-1}.h^{-1}$, was observed from butyrate with a newly isolated photosynthetic non-sulfur *Rhodobacter sphaeroides* strain ZX-5 (Tao *et al.*, 2008). Overall, when compared to biophotolysis processes, the photofermentative hydrogen rates are higher. Moreover, this photo-fermentation bioprocess offers the possibility of combining dark fermentation processes to improve significantly the overall hydrogen yield (see I-2.2.3).

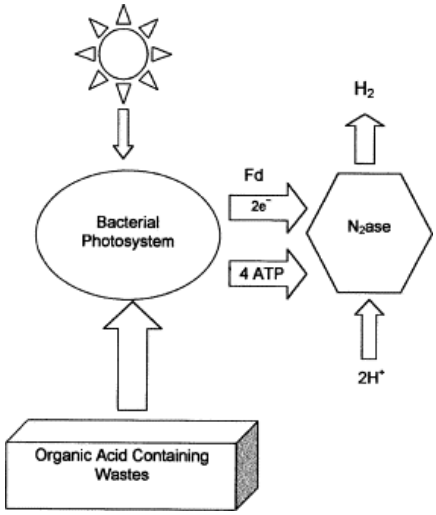


Figure I-4: Principles of photofermentation by photosynthetic bacteria (from Hallenbeck and Benemann, 2002)

I.1.2.4 **Dark fermentation processes**

The last biological way to produce hydrogen is dark fermentation, in which carbohydrate-rich substrates are decomposed into metabolic end-products such as volatile fatty acids, alcohols, organic acids and into a mixed biogas containing H_2, CO_2, by strict and/or facultative anaerobic bacteria (Levin *et al.*, 2004; Mudhoo *et al.*, 2011). Pure fermentative cultures such as *Clostridium sp.* (Jo *et al.*, 2008), *Escherichia coli* (Yoshida *et al.*, 2005) and *Enterobacter* sp. (Shin *et al.*, 2007) have largely been investigated. Mixed culture dark fermentation processes have recently gained more attention to produce hydrogen, since they better adapt to environmental changes, are capable to promote low cost renewable organic waste, and exhibit high resistance and resilience of the microbial communities against operation changes (Quéméneur *et al.*, 2011). Producing hydrogen by dark fermentation in mixed culture correspond to the early stages of anaerobic digestion, an ubiquitous biological process to convert organic matter to methane and carbon dioxide occurring under strict anaerobic conditions.

Figure I-5 illustrates the microbial pathways occurring in anaerobic digestion, including H_2 evolution. The first step concerns the hydrolysis of complex organic matter to small

polymers or soluble molecules available for acidogens. The acidogenic bacteria in the subsequent step convert simple carbohydrates, proteins and fats to VFAs, alcohols, metabolic end-products and to CO_2 and H_2. Hydrogen is accumulated as a key intermediate and can be rapidly consumed by other microorganisms in mixed cultures, such as homoacetogens, methanogens, and sulfate-reducing bacteria (

Figure I-5) (Chen *et al.*, 2008a; Das and Veziroglu, 2008; Valdez-Vazquez, 2009).

In comparison with other biological techniques for hydrogen production, dark fermentation processes takes the advantages of getting rid of light requirement, which allows its operation all day long, and largely reduces the land requirement of the overall chain of production. Moreover, dark fermentation combines valuable molecule generations (*e.g.* H_2 and VFAs) as well as waste volume reduction. Indeed, the soluble metabolic products can be further used as value-added chemical materials such as for the production of bioplastics. The difficulty referred to dark fermentation is that hydrogen performances of the bioprocesses are highly dependent on the metabolic and phylogenetic interactions occurring within the microbial communities that are difficult to control. Further researches are still required to select suitable hydrogen producing cultures, to control their activity under optimal operating parameters and to design reliable and stable process.

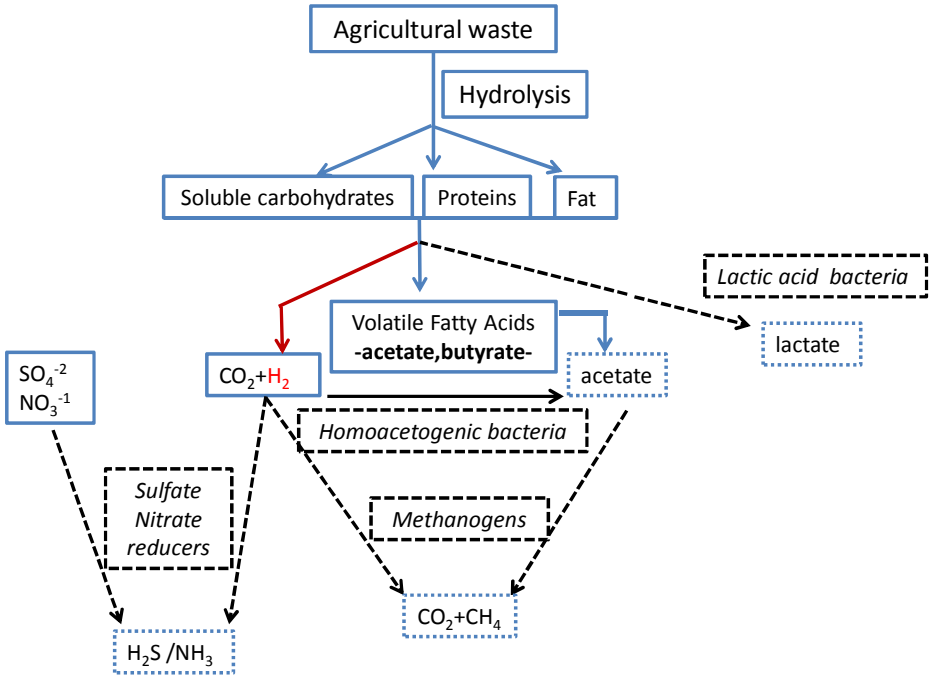


Figure I-5: Microbial anaerobic pathways in microbial ecosystems degrading organic waste. Bold arrows indicate hydrogen-producing pathways and dotted arrows hydrogen-consuming pathways (Guo *et al.*, 2010).

I.1.3 Main microbial pathways producing biohydrogen by dark fermentation

In order to understand the metabolic pathways involved in hydrogen production by mixed culture, glucose or sucrose as model substrates have been largely investigated and their pathway are now well described. As showed in Figure I-6, the first step concerns glucose or pentose conversion to pyruvate followed by the glycolysis metabolic pathways (or Embden-Meyerhoff-Parnas), in which adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) are generated as energy storage and electron donor for cellular synthesis, respectively. Pyruvate, as a key product of the central metabolism,

can be then converted to acetyl-CoA, reduced ferredoxin and CO_2. The excessive reduced ferredoxin is then re-oxidized to form hydrogen with the help of hydrogenases. In some enteric bacteria, such as *Escherichia coli*, the pyruvate can be directly converted to acetyl-CoA, and thereafter formate which generates hydrogen and CO_2 with the help of a formatelyase. Acetyl-CoA is a central intermediate of the butyrate, acetate and ethanol fermentation pathways occurring according to the species of microorganisms, the physiological state of the microorganisms, the operational conditions, the type of substrates and the organic load. The excessive reduced NADH is re-oxidized to form hydrogen with the help of hydrogenases. Among all metabolic possibilities, acetate and butyrate fermentation occurs more easily since they are associated with ATP and reduced ferredoxin and NADH generation that are strongly required for cell anabolism (Latrille *et al*., 2011)

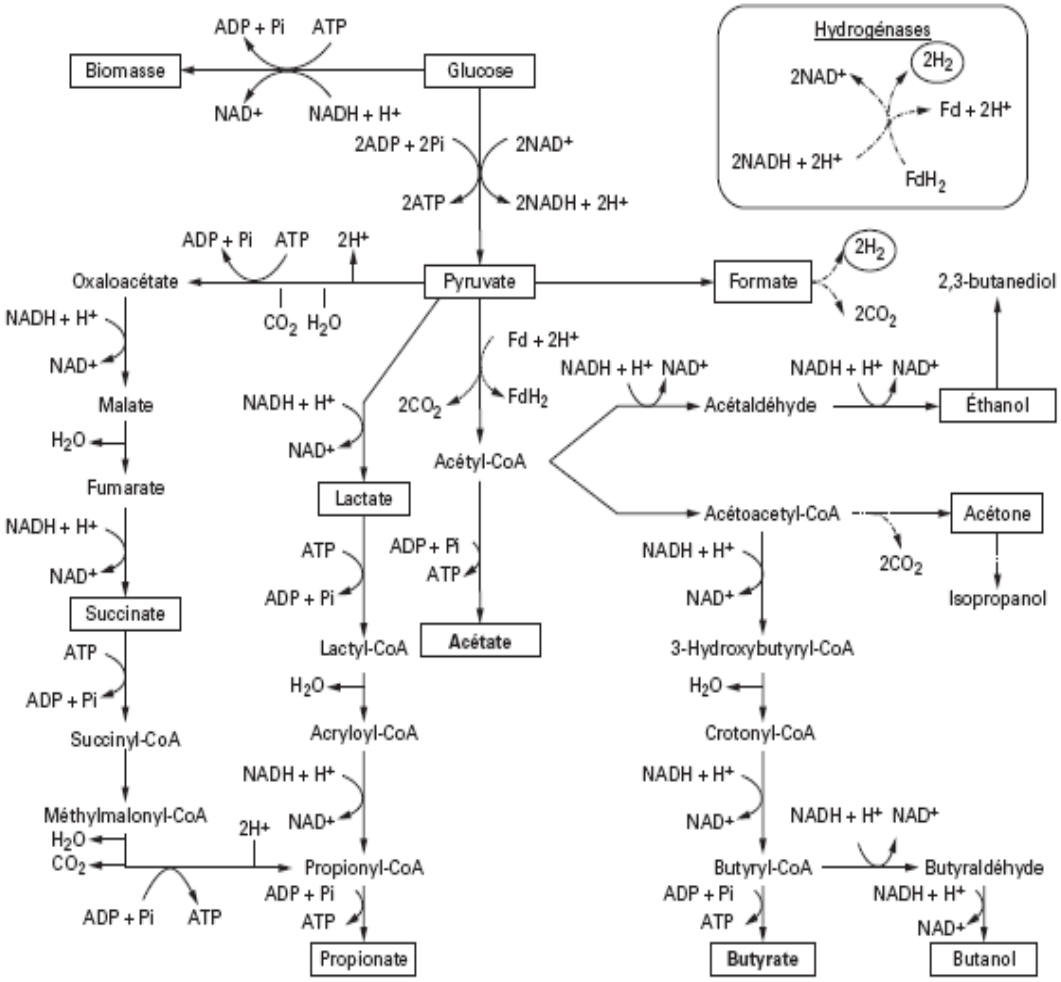


Figure I-6: Main metabolic pathways of biohydrogen production by dark fermentation (according to Latrille *et al.*, 2011)

Within the large range of end products generated by the various microbial metabolisms occurring in mixed cultures, both acetate and butyrate accumulate substantially with the main hydrogen producing metabolisms as described in *Clostridium* sp. or *Enterobacteriacae* (Eqs. (I-1) and (I-2)).

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G_o' = -215\ kJ.mol^{-1} \qquad (I\text{-}1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \Delta G_o' = -264\ kJ.mol^{-1} \qquad (I\text{-}2)$$

Accumulating acetate as sole end product gives a stoechiometric production of 4 $mol_{H2}.mol_{hexose}^{-1}$, equivalent to 498 $ml_{H2}.g_{hexose}^{-1}$ (0 °C, 1atm.), while in the butyrate pathway, a lower molar hydrogen yield is observed with 2 $mol_{H2}.mol_{hexose}^{-1}$, equivalent to 249 $ml_{H2}.g_{hexose}^{-1}$ (0 °C, 1atm) (Hawkes *et al.*, 2007).

However, the accumulation of acetate in the medium does not necessarily imply higher biohydrogen production since several microbial species can also convert hydrogen and carbon dioxide to acetate (Eq. (I-3)) (Antonopoulou *et al.*, 2008).

$$2CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{(I-3)}$$

In mixed cultures, a ratio of 3:2 of butyrate / acetate is usually observed, resulting in a theoretical average hydrogen yield of 2.5 $mol_{H2}.mol_{hexose}^{-1}$ (Hawkes *et al.*, 2007). Hawkes *et al.* (2007) even proposed a theoretical equation that could represent an average metabolism occurring in mixed cultures (Eq.(I-4)):

$$4\ C_6H_{12}O_6 + 2\ H_2O \rightarrow 3\ CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10\ H_2 \quad \Delta G_o' = -252\ kJ.mol^{-1} \qquad \text{(I-4)}$$

Other studies suggested a possible metabolite way to convert glucose to acetate and ethanol with a hydrogen yield of 2 $mol_{H2}.mol_{glucose}^{-1}$ (Eq.(I-5)) (Rodriguez *et al.*, 2006)

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 \quad \Delta G_o' = -225\ kJ.mol^{-1} \qquad \text{(I-5)}$$

In mixed cultures, other metabolic end-products such as propionate and caproate, may also accumulate. Propionate is a metabolite of a hydrogen-consuming pathway. Propionate is an alternative pathway of glucose degradation where no H_2 is generated in presence of propionic acid bacteria. In contrast, caproate is generated after direct consumption of hydrogen and butyrate and/or acetate (Eqs. (I-6), (I-7) and (I-8)). Finally, the lactate pathway carried by lactic acid bacteria (LAB) such as *Streptococcus* sp., *Lactobacillus* sp. redirects the electronic flow from glucose to lactate formation instead of acetate and H_2 (Eqs.

(I-9), (I-10)). Because of direct competition on the same substrate, part of the glucose pathway going to lactate accumulation does not produce excess in reducing equivalent and therefore no bioH_2.

$C_6H_{12}O_6 + 2\ H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$ $\Delta G_o' = -359\ kJ.mol^{-1}$ (I-6)

$CH_3(CH_2)_2COOH + CH_3COOH + 2H_2 \rightarrow CH_3(CH_2)_4COOH + 2H_2O$ $\Delta G_o' = -48\ kJ.mol^{-1}$ (I-7)

$CH_3(CH_2)_2COOH + 2CO_2 + 6H_2 \rightarrow CH_3(CH_2)_4COOH + 4H_2O$ $\Delta G_o' = -143\ kJ.mol^{-1}$ (I-8)

$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2$ $\Delta G_o' = -117\ kJ.mol^{-1}$ (I-9)

$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + CH_3CH_2OH + CO_2$ $\Delta G_o' = -216\ kJ.mol^{-1}$ (I-10)

I.1.4 Involvement of hydrogenases as electron and proton flux regulators

The production of hydrogen in many microorganisms is intimately related to their energy metabolism. Hydrogenases and nitrogenases are the two main known enzymes categories involved in hydrogen generation. The kinetic constant of hydrogenase is 6000-9000 s^{-1} and much higher than the kinetic constant of nitrogenases that is only of 6-9 s^{-1} (Melis and Melnicki, 2006). Nitrogenase enzymes are usually responsible for nitrogen-fixation with a low affinity to their substrates, and hydrogen production only occurs when the nitrogen source is limited (Das *et al.*, 2006; Yu and Takahashi, 2007). Nitrogenase-catalysed hydrogen production showed that nitrogenases use a great amount of energy and lead to low biomass productivity (Eq.(I-11)) (Das *et al.*, 2006).

$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$ (I-11)

Hydrogenases are therefore more than enzymes that only catalyse biohydrogen generation, but act also as one of the specific mechanisms to dispose or regulate the excess of electrons and protons in the cells to/from H_2. Indeed, this reaction is reversible (Eq.(I-12))

$2H^+ + 2e- \leftrightarrow H_2$ (I-12)

In presence of H_2 and an electron acceptor, hydrogenase enzymes can act as a H_2 uptake enzyme coupled to the reduction of electron acceptors such as oxygen, nitrate, sulfate, carbon dioxide and fumarate (Das *et al.*, 2006). In contrast, hydrogenases are involved in H_2 evolution by reducing protons in presence of an electron donor at low potential such as reduced ferredoxin or NADH (Das *et al.*, 2006).

I.1.4.1 **Structures, localizations and functions of hydrogenases**

Hydrogenases are classified into three distinct classes according to their metal active sites: nickel-iron ([NiFe]-hydrogenases) or iron-only ([FeFe]-hydrogenases). In some [NiFe] hydrogenases, one of the Ni-bound cysteine residues is replaced by a selenocysteine, and hydrogenases are thus called [Ni-Fe-Se]-hydrogenases. Based on protein sequence homology of thirty sequenced microbial hydrogenases, Das *et al.* (2006) concluded that [NiFe]- and [Ni-Fe-Se] hydrogenases are homologous and share the same evolutionary origin. Recently, mononuclear Fe active site hydrogenases were described in methanogens with no iron-sulfur clusters, and so named as [Fe]-hydrogenases (Lyon *et al.,* 2004). [NiFe]-and [FeFe]-hydrogenases present several structural and functional differences: as showed in Figure I-7, the [NiFe] hydrogenases are bound to the polypeptidic chain through the thiolate functions of four cysteine residues, while the [Fe-Fe] hydrogenases have just one single protein ligand to a cysteine. The [NiFe] hydrogenases are heterodimeric proteins consisting of small subunit of about 30 kDa containing one to three iron-sulfur clusters associated to a large subunit of about 60 kDa containing the nickel-iron active site. In contrast, the [FeFe]-hydrogenases are monomeric proteins with a wide size range from 45 kDa to 120 kDa (Meyer, 2007; Mulder *et al.*, 2011). Although [NiFe]-and [FeFe]-hydrogenases exhibit structural differences, some features in their structures are common since they all contain a various number of Fe-S clusters surroundings their active site. These [Fe-S] clusters are

considered as accessory domains which connect to redox proteins such as ferredoxins or NADH-ubiquinone-oxidoreductase.

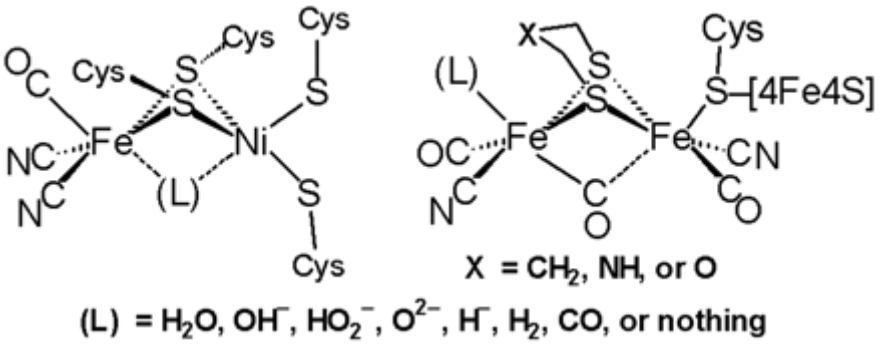


Figure I-7: Consensus structures of the [NiFe]-and [FeFe]-hydrogenases active sites. (from Tey and Hall, 2005).

In terms of enzyme locations, the [NiFe] hydrogenases are overspread in the cell either as membrane-bound elements or as free enzymes in cytoplasmic and periplasmic cellular compartments for the production or uptake of hydrogen, respectively. The [NiFeSe] hydrogenases are membrane-bound or located in the cytoplasm and exhibit an activity of hydrogen oxidation. [NiFe] hydrogenases are synthesized by anaerobic bacteria, photosynthetic bacteria, cyanobacteria sulphate-reducing bacteria and methanogens (Das *et al.*, 2006). The [FeFe] hydrogenases are mostly abundant in the cytoplasmic and periplasmic compartment as well as in chloroplasts. So far, the [FeFe]-hydrogenases have been only reported to present hydrogen generation activity in photosynthetic bacteria, anaerobic fermentative bacteria, cyanobacteria, green algae and protozoa (Das *et al.*, 2006; Valdez-Vazquez *et al.*, 2009).

I.1.4.2 **Factors influencing the hydrogenase activity**

The impact of environmental factors on hydrogenase activity in real conditions of production has been poorly investigated. Valdez-Vazquez *et al.* (2009) reported that the

hydrogenase activity (uptake and evolution) remains very low in cells of *Clostridium beijerinckii* maintained at a pH lower than 5.2, whereas hydrogenase activity in *Clostridium acetobutylicum* was higher at pH 5.8 than at 4.5 (Valdez-Vazquez *et al.*, 2009). This is consistent with observations in cellular cultures where the optimal pH for bioH_2 production by dark fermentation is around 5.5 (see I-2.1.1). Basically, the *in vitro* hydrogenase activity follows an Arrhenius law within the range of 15-50 °C (Adams and Mortenson, 1984). Together with *in vivo* observations, an optimal temperature of 50 °C was suggested by Valdez-Vazquez *et al.* (2009). This is also in accordance with other observations in engineered processed reporting higher hydrogen production rates under thermophilic than mesophilic conditions (Shin *et al.*, 2004; Valdez-Vazquez *et al.*, 2006).

Considering the essential role of the iron element in all kinds of hydrogenases, the impact of Fe^{2+} concentration in the culture medium has also been investigated (Yang and Shen, 2008; O-Thong *et al.*, 2008a; Wang *et al.*, 2008). No agreement on the optimal iron concentration for hydrogen fermentation was found, considering the large range of optimal Fe^{2+} concentrations found in the literature, *i.e.* ranging from 10 $mg.L^{-1}$ to 590 $mg.L^{-1}$ (Table I-1). Such differences are probably due to the complex composition of the microbial community combined with the variability of the applied operational parameters. Interestingly, all these studies were carried out in batch reactors, which allowed the microbial consortium to ferment feedstock when placed under optimal experimental conditions. But, so far, no data has been reported about the iron concentration effect on the microbial selection that could occur in continuous processes for H_2 production.

Table I-1: Optimal Fe^{2+} concentration published for hydrogen production in dark fermentation processes.

Substrate	Inoculum	Reactor	Studied range of Fe^{2+}concentration ($mg.L^{-1}$)	Optimal Fe^{2+} concentration ($mg.L^{-1}$)	References
Glucose	Digested sludge	Batch	0-1500	350	Wang *et al.*, 2008
Sucrose	Anaerobic sludge	Batch	0-1764	353	Lee *et al.*, 2001
Sucrose	Cracked cereals	Batch	0-1842	590	Zhang *et al.*, 2005
Starch	Cracked cereals	Batch	1.2-100	10	Liu and Shen, 2004
Starch	Anaerobic sludge	Batch	0-1474	55	Yang and Shen , 2008
Food waste	Grass compost	Batch	0-250	132	Lay *et al.*, 2005
Palm oil mill effluent	Anaerobic sludge	Batch	2-400	257	O-Thong *et al.*, 2008a

I.1.5 **Potentiality of available feedstock for bioH_2 production**

Many studies investigating hydrogen production by dark fermentation have used simple sugars such as glucose or sucrose as model substrates. A review made by Li and Fang (2007) reported over 98 studies dealing with hydrogen production by dark fermentation. Among then, 66 % are related to simple sugars or synthetic wastewaters mainly composed of carbohydrates (Li and Fang, 2007). Hydrogen yields reported from glucose ranged 0.89 $mol_{H2}.mol_{glucose}^{-1}$ to 2.30 $mol_{H2}.mol_{glucose}^{-1}$. The highest hydrogen yield on carbohydrates was obtained from sucrose *i.e.* 2.68 $mol_{H2}.mol_{glucose}^{-1}$, while the lowest hydrogen yield was observed from cellulose, *i.e.* 0.4 $mol_{H2}.mol_{glucose}^{-1}$. Recently, Quéméneur *et al.* (2011) showed that the chain length of carbohydrates can influence the hydrogen yields. In this study, decreasing hydrogen yields were observed from monomeric (fructose) to trimeric units (maltotriose) of simple sugars. Furthermore, it was shown that the type of linkage between the sugar units had also an effect on hydrogen yields (Quéméneur *et al.*, 2011). Unfortunately, to date, only little is known about the impact of the structural properties of complex organic waste. Indeed, only

fewer studies have looked to solid substrate conversion for bioH_2 production. Amongst all the organic materials to be potentially used as substrates for sustainable biohydrogen production, the most interesting ones must not only be abundant and readily available but, also, cheap and highly biodegradable. Agri-waste and food waste meet all these requirements. As to their abundance, about 0.7 billion tons of agricultural and forestry waste were generated in Western Europe between 1998 and 2001 (European community, 2003). In France, a survey of the years 1995 to 2006 showed that total annual waste production had increased to about 849 million tons by 2006, of which agricultural and forestry waste represented around 43 %, *i.e.* 374 million tons (Dubois, 2006). In Germany, the second biggest agricultural country in Europe, agri-waste represented more than 175 million tons per year in 2000, including 25 million tons per year of agricultural biomass. By way of comparison, German municipal waste represented only 16 million tons per year and industrial waste 9 million tons (Weiland, 2000).

Three categories of agricultural residues can thus be distinguished: (i) waste generated from direct agricultural production, i.e. crop residues; (ii) livestock waste, i.e. animal manure, and (iii) food waste. A state of the art on the use of these kinds of waste will be discussed in the following paragraphs as well as their potentiality for generating biohydrogen by dark fermentation. Hydrogen yield is a criterion to estimate the efficiency of conversion from different waste substrates, defined as the amount of hydrogen produced per amount of substrate added, degraded or consumed.

I.1.5.1 **Crop Residues**

Agricultural residues from harvested crops are the most abundant, cheapest and most readily available organic waste to be biologically transformed; they include straw, stover, peelings, cobs, stalks, bagasses, and other lignocellulosic residues (Mtui, 2009). The annual lignocellulosic biomass generated by the primary agricultural sector has been evaluated at approximately 200 billion tons worldwide (Ren *et al.*, 2009). All agricultural crops are

biodegradable and, to varying degrees, may be converted biologically in anaerobic digestion processes to biohydrogen and biomethane.

Hydrogen yields from various crop substrates, as recorded in the literature, are presented in Table I-2. The origins of the organic substrates are quite similar; nevertheless, untreated raw material presents generally lower yields, ranging from 0.5 to 16 $mL_{H2}.g_{VS}^{-1}$. Under mesophilic conditions the lowest yield was reported from the conversion of wheat straw to hydrogen in a batch reactor (Fan *et al.*, 2006b), while the highest was obtained using cornstalks (Li *et al.*, 2007). The yield of fermentative hydrogen from crop residues in thermophilic conditions at 70 °C was higher than that in mesophilic conditions indicating that temperature favours hydrolysis (Karlsson *et al.*, 2008). Indeed, the "cornstalks" category in Table I-2 shows variable hydrogen yields, likely because of the varied composition in carbohydrates, including cellulose and hemicellulose, and in lignin (Li *et al.*, 2007; Zhang *et al.*, 2007a). Moreover, as reported in anaerobic digesters producing methane from agricultural waste, the crop species, the harvesting time and the variable silage period must all be considered as main factors impacting on biogas fermentation (Amon *et al.*, 2007). A recent review of the literature summarized the composition of different crops residues, *e.g.* wheat straw, corn stover and rice straw as containing cellulose, hemicelluloses and lignin in a range of approx. 32-47 %, 19-27 % and 5-24 %, respectively (Saratale *et al.*, 2008). Although no trend was observed in the reported data, a reasonable hypothesis is that biohydrogen yields may be inversely correlated to the cellulose and lignin contents of the waste, as observed by Buffiere *et al.* (2006) for methane production.

The production of biohydrogen from crop waste biomass is limited by the hydrolytic activity of the microorganisms involved in the biological attack of the heterogeneous and microcrystalline structure of lignocellulosic component, and in the decomposition of cellulose-like compounds to soluble sugars. Appropriate pretreatment steps for the raw material are often required in order to favour hydrolysis. The main pretreatments are based on mechanical, physical, chemical and biological techniques (Mtui, 2009; Monlau *et al.*, 2012).

A mechanical shredding step is essential to reduce particle size and increase the surface area of the organic waste prior to fermentation. As a consequence, solubility and fermentation efficiency are both favoured during the acidogenic fermentation step. In all studies reported in Table I-2 the crop residues were mechanically treated prior to the experiments and this technique should be further investigated to determine the influence of such pretreatment on overall performances. Chemical pretreatments methods using oxidizing agents, alkali, acids and salts are most frequently investigated because they require no direct energy input (Mtui, 2009). The biohydrogen yield from cornstalks treated by NaOH (0.5 %) reached 57 $mL_{H2}.g_{VS}^{-1}$, *i.e.* 19-fold the initial value of raw material (3 $mL_{H2}.g_{VS}^{-1}$) (Zhang *et al.*, 2007a). Zhang *et al.* (2007a) also investigated biohydrogen production from cornstalk waste after an acidification pretreatment coupled to heat pretreatment. A maximum cumulative H_2 yield of 150 $ml_{H2}.g_{VS}^{-1}$ was obtained after a 0.2 % HCl treatment, *i.e.* 50 times the initial value, thus proving the efficiency of the acidification pretreatment step (Zhang *et al.*, 2007b). Although this value is remarkable in the light of the average values reported in Table I-2, such performances are within the range of the theoretical biohydrogen yield in mixed cultures, *i.e.* 311 $mL_{H2}.g_{\ hexose}^{-1}$, calculated from 2.5 $mol_{H2}.g_{Hexose}^{-1}$ according to Hawkes *et al.* (2007). Fan *et al.* (2006b) demonstrated that an acidic pretreatment of 2 % HCl coupled to microwave heating led to the increase of soluble sugar content of wheat straw from 0.2 % to 9.6 % and to the decrease of cellulose and hemicellulose content from, respectively, 22 % to 15 % and 21 % to 13 %. The maximum hydrogen yield observed in this case was 68 $mL_{H2}.g_{VS}^{-1}$, which is 136 times the initial value (0.5 $mL_{H2}.g_{VS}^{-1}$) observed on untreated material (Fan *et al.*, 2006b). Similar results were observed with steam explosion as pretreatment, with a yield increasing from 9 $mL_{H2}.g_{\ raw\ corn\ straw}^{-1}$ to 68 $mL_{H2}.g_{\ treated\ corn\ straw}^{-1}$ (Li *et al.*, 2007). Given the present state of knowledge, further experimentation is required to better understand the impact on biohydrogen production performances of the compositions and characteristics of organic substrates. Pretreatment processes for crop residues also require specific investigation since the origins and compositions of the organic substrates determine which specific pretreatment is the most suitable.

I.1.5.2 **Animal manure – livestock waste**

Three main types of animal manure have been distinguished: urinary waste i.e. slurry or liquid manure from livestock or poultry; solid manure or farm yard manure; and wastewater which is a collection of process water in farms, feedlot runoff, silage juices, bedding, disinfectants and liquid manure (Burton and Turner, 2003). More than 1500 million tons of animal manure is produced yearly, including 1284 million tons of cattle manure and 295 million tons of pig manure across the 27 member states of the European Union (Holm-Nielsen *et al.*, 2009). Where manure is not managed or treated, it represents a major risk of air and water pollution. On the one hand, nutrient leaching (primarily nitrogen and phosphorous) and pathogen contamination can lead to direct surface water damage and, on the other hand, manure can release up to 18 % CO_2 equivalent and 37 % CH_4, contributing to the greenhouse effect (Holm-Nielsen *et al.*, 2009).

On European farms, animal manure is usually treated in storage tanks, and then the liquid fraction is separated by centrifugation and finally spread on farmland. The solid fraction is subsequently treated by anaerobic digestion to be further used as fertilizer in agriculture (Moller, 2006). Since agricultural biogas facilities have been extensively used to co-digest manure and other residues suitable for methane production, these large-scale farm installations provide the necessary equipment to readily implement biohydrogen bioprocesses (Cantrell *et al.*, 2008).

Biohydrogen yields from livestock waste are presented in Table I-2. Mainly, they are much lower than those observed from crop residues, with values ranging from 4 to 29 $mL_{H2}.g_{VS}^{-1}$. In most studies, either chemical or thermal pretreatment associated to thermophilic conditions are required to avoid methanogenic activity. Indeed, the indigenous methanogenic microflora will rapidly convert hydrogen to methane, as shown by Yokoyama *et al.* (2007a). The highest yield (*i.e.* 65 $mL_{H2}.g_{VS}^{-1}$) was reported in a study investigating the potential for hydrogen production of cattle manure thermally pretreated (Table I-2). This high

yield was likely the result of using fresh manure sampled directly at the cattle feedlot prior to the experiment. This assumption is supported by the study of Bonmati *et al.* (2001) who observed a 3.5-fold decrease in methane production when the pig slurry was stored for several months. Meanwhile, the ammonium concentration increased 3-fold over the initial value because of the decomposition of organic matter (Bonmati *et al.*, 2001). A similar inhibition has been observed for biohydrogen production from animal slurry. Indeed, Kotsopoulos *et al.* (2009) concluded that the low production yield of 4 $mL_{H2}.g_{VS}^{-1}$ from pig slurry was due to ammonium inhibition. Livestock manure from pork and poultry have been reported to contain up to 4g $N.L^{-1}$ and cattle manure about 1.5 g $N.L^{-1}$ (Angelidaki and Ahring, 1994). Because of the high nitrogen content, shock loading of slurry can cause severe inhibition of the whole biological anaerobic and hydrogen fermentation processes (Hobson *et al.*, 1974; Salerno *et al.*, 2006). Additionally, it has also been observed that high sulfate concentrations in swine manure act as a strong inhibitor of biohydrogen production through the growth of highly competitive hydrogen-consuming sulfate-reducing bacteria (Chen *et al.*, 2008b). With the aim of avoiding nitrogen inhibition, another study on liquid swine manure showed a high yield of 209 $mL_{H2}.g_{VS}^{-1}$ after the addition of glucose as an additional substrate in a semi-continuously-fed reactor (Zhu *et al.*, 2009). This observation suggests the potential use of the co-digestion of animal manure and carbohydrate-rich feed to produce biohydrogen. In this case, the co-digestion process should even be envisaged locally, in the light of agricultural facilities to directly use local crop materials, in order to optimize the loading ratio C/N by dilution of other inhibiting factors. This should, consequently, increase the stability of the biological process. A recent study investigating the anaerobic co-digestion of cattle slurry with vegetable/fruit wastes and chicken manure showed a substantial 2-fold increase in the methane yield (Callaghan *et al.*, 2002).

I.1.5.3 **Food waste**

Food waste has high energy content and is highly biodegradable, *e.g.* it contains 85-95 % of volatile solids and 75-85 % moisture, favouring microbial development (Li *et al.*, 2008).

Food waste is usually disposed as landfill which can lead to problems of putrid smells and leachates polluting underground water if not handled properly (Cantrell *et al.*, 2008). Anaerobic digestion is recommended for treating food wastes (Ward *et al.*, 2008). Over the last decades food waste has been the most studied feedstock for hydrogen production, including kitchen refuse (Jayalakshmi *et al.*, 2009), a part of municipal waste (Eroglu *et al.*, 2009), food industry co-products such as oil mill (Eroglu *et al.*, 2009; O-Thong *et al.*, 2008a), cheese whey (Venetsaneas *et al.*, 2009), and starch-manufacturing waste (Yokoi *et al.*, 2002). In Table I-2, several maximal biohydrogen production yields observed in anaerobic reactors are reported. As for the results obtained with crop residues and livestock waste, the performances display great variation, from 3 $mL_{H2}.g_{VS}^{-1}$ to more than 290 $mL_{H2}.g_{VS}^{-1}$, due to the different composition of the matter involved. The average production is substantially higher than the values obtained from crop residues and livestock. About ten years ago, individual food substrates *i.e*. rice, carrot, cabbage, chicken skin, egg and lean meat began to be sorted out from municipal waste for assessment (Okamoto *et al.*, 2000). In the latter study, biohydrogen production was assessed from a range of relatively simple substrates for further assessment of the production potential with mixtures made up of such simple constituents. Later, other studies using food waste from institutional catering were carried out in batch tests and showed yields of 60 $mL_{H2}.g_{VS}^{-1}$ to 196 $mL_{H2}.g_{VS}^{-1}$ (Kim *et al.*, 2004; Li *et al.*, 2008). Studies of continuous fermentation systems have been reported more recently, showing no significantly higher yield, but they have proved the feasibility of using food waste in future continuous pilot or industrial-scale applications (Karlsson *et al.*, 2008; Wang *et al.*, 2009b). Again, more recently, many studies have focused on agri-food industry waste as a source of substrates for producing biohydrogen (Aceves-Lara *et al.*, 2008; O-Thong *et al.*, 2008a; Eroglu *et al.*, 2009; Venetsaneas *et al.*, 2009; Venkata Mohan *et al.*, 2009). Among them, carbohydrate-rich waste shows great promise for the intensive production of biohydrogen. For instance, biohydrogen yields from molasses and cheese whey approached a value of 2.5 $mol_{H2}.mol_{hexose}^{-1}$ which corresponds to the maximal expected yield in mixed cultures (Aceves-Lara *et al.*, 2008; Venetsaneas *et al.*, 2009).

In addition, thermophilic conditions also favour biohydrogen production. Indeed, food waste from institutional catering generated around 81 $mL_{H2}.g_{VS}^{-1}$ under thermophilic conditions, compared to 63 $mL_{H2}.g_{VS}^{-1}$ under mesophilic conditions (Kim and Shin, 2008). Other studies reported increasing yields from 13 $mL_{H2.}g_{VS}^{-1}$ to 65 $mL_{H2}.g_{VS}^{-1}$, respectively under mesophilic and thermophilic conditions (Karlsson *et al.*, 2008; Wang and Zhao, 2009). For the lowest values, *i.e*. 12.6 $mL_{H2.}g_{VS}^{-1}$, a mixture of slaughterhouse waste, food waste and manure was utilized as substrate. It included much proteins and fat (Karlsson *et al.*, 2008), which might well explain of the low hydrogen yield. Although thermophilic conditions are recommended, they are energy consuming. If the energy for heating the fermentation system could be generated through a biogas/thermal exchange system, thermophilic continuous processes could then be considered as sustainable.

Table I-2: Estimated H_2 production yields of anaerobic reactors treating agricultural waste.

Substrate	Maximum assessed production yield ($mlH_2.g_{VS}^{-1}$)	Pretreatment*	Temperature (°C)	Reactor operation mode	Reference
Corn straw	9	-	35	Batch	Li *et al.*, 2007
Corn straw	68*	1.5MPa10min	35	Batch	Li *et al.*, 2007
Corn stover	49*	220 °C 3min	35	Batch	Datar *et al.*, 2007
Corn stover	66*	1.2 % HCl+200 °C 1min	35	Batch	Datar *et al.*, 2007
Cornstalk	3	-	36	Batch	Zhang *et al.*, 2007a
Cornstalk	57	0.5 % NaOH	36	Batch	Zhang *et al.*, 2007a
Cornstalk	150	0.2 %HCl boiled 30min	36	Batch	Zhang *et al.*, 2007a
Grass silage	6	-	35	Batch	Karlsson *et al.*, 2008
Grass silage	16	-	70	Batch	Karlsson *et al.*, 2008
Maize leaves	18	-	70	Batch	Ivanova *et al.*, 2009
Maize leaves	42	130 °C 30min	70	Batch	Ivanova *et al.*, 2009
Rice bran	61	n.d.	35	Batch	Noike and Mizuno, 2000
Sweet sorghum plant	32.4*	130 °C 30min	70	Batch	Ivanova *et al.*, 2009
Sugarcane bagasse	19.6*	130 °C 30min	70	Batch	Ivanova *et al.*, 2009
Silphium trifoliatum leaves	10.3*	130 °C 30min	70	Batch	Ivanova *et al.*, 2009
Rice straw	24.8	-	55	Batch	Chen *et al.*, 2008b
Wheat straw	1	-	36	Batch	Fan *et al.*, 2006b
Wheat straw	68	HCl 2 %+microwave heating	36	Batch	Fan *et al.*, 2006b

Wheat straw	49*	130 ℃ 30min	70	Batch	Ivanova *et al.*, 2009
Wheat bran	43	n.d.	35	Batch	Noike and Mizuno, 2000
Cow feces and urine	18*	-	75	Batch	Yokoyama *et al.*, 2007a
Cow feces and urine	29*	-	60	Batch	Yokoyama *et al.*, 2007a
Cow feces and urine	0.7*	-	37	Batch	Yokoyama *et al.*, 2007a
Cattle wastewater	53*	-	45	Batch	Tang *et al.*, 2008
Dairy manure	18	0.2 %HCl boiled 30min	36	Batch	Xing *et al.*, 2010
Dairy manure	14	0.2 %NaOHboiled30 min	36	Batch	Xing *et al.*, 2010
Dairy manure	14	infrared radiation 2h	36	Batch	Xing *et al.*, 2010
Pig slurry	4	-	70	CSTR	Kotsopoulos *et al.*, 2009
Swine liquid manure	209*	-	35	Semi-contino usly -fed fermeter	Zhu *et al.*, 2009
Rice	96	-	35	Batch	Okamoto *et al.*, 2000
Carrot	71	-	35	Batch	Okamoto *et al.*, 2000
Cabbage	62	-	35	Batch	Okamoto *et al.*, 2000
Chicken skin	10	-	35	Batch	Okamoto *et al.*, 2000
Egg	7	-	35	Batch	Okamoto *et al.*, 2000
Lean meat	8	-	35	Batch	Okamoto *et al.*, 2000
Foodwaste	196	160 ℃ 2h	36	Batch	Li *et al.*, 2008
Foodwaste	60*	n.d.	35	Batch	Kim *et al.*, 2004
Foodwaste	77	-	35	Batch	Lay *et al.*, 2005
Foodwaste	104.79	-	30	Batch	Sreela-or *et al.*,2011

Foodwaste	125*	-	35	CSTR	Shin and Youn, 2005
Foodwaste	63	pH12.5 1day	35	ASBR	Kim *et al.*, 2008
Foodwaste	65	-	40	Demi-continuous rotating drum	Wang *et al.*, 2009b
Foodwaste	13	-	20	CSTR	Karlsson *et al.*, 2008
Foodwaste	3	-	37	CSTR	Karlsson *et al.*, 2008
Foodwaste	16.5	-	55	CSTR	Karlsson *et al.*, 2008
Kitchen waste	72	-	n.d.	Inclined plug flow reactor	Jayalakshmi *et al.*, 2009
Molasses	2.5 $molH_2/molsucrose$	-	37	CSTR	Aceves-Lara *et al.*, 2008
Molasses	2.1mol_{H2}/mol_{hexose}	-	35	CSTR	Chang *et al.*, 2008a
Sweet lime peelings extracts	76.4ml/g CODr*	121 ℃ pH=7 40min	32	Batch	Venkata Mohan *et al.*, 2009
Bean curd manufacturing waste	21	n.d.	35	CSTR	Noike and Mizuno, 2000
Cheese whey	290*	$NaHCO_3$ 20g/L	35	CSTR	Venetsaneas *et al.*, 2009
Palm oil mil effluent	84.4*	-	60	Batch	O-Thong *et al.*, 2008a

(*calculated from literature data, - no pretreatment of feedstock, n.d. not determined)

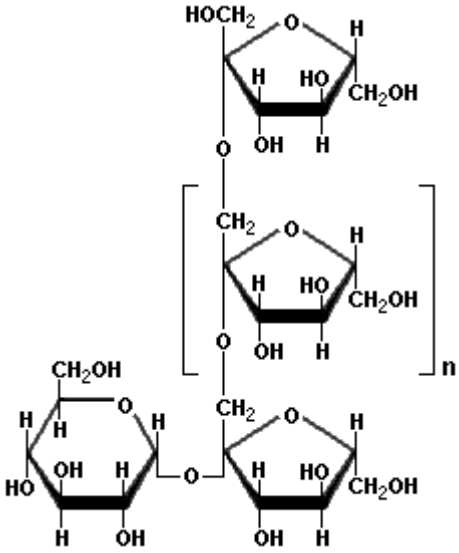
I.1.5.4 **A model substrate: the Jerusalem artichoke plant**

In this study, the Jerusalem artichoke was selected as a model raw substrate for dark fermentation hydrogen production, for its high content in carbohydrates and its low fertilization demand on soils. Moreover, the type of carbohydrate that accumulates in the Jerusalem artichoke corresponds to fructose polymers, *i.e*. inulin that has been poorly investigated for biohydrogen production. Historically, the Jerusalem artichoke is native to North America from a large area from the Great Lakes to north, Arkansas to south, Mississippi river to west and Piedmont coastal plain to east. This plant was transferred to Europe and cultivated in Rome in the 16th century. The tubers of this plant was recorded in early human consumption history as raw radish and also cooked in soup (Kosaric *et al.*, 1984). This plant has been cultivated because of several remarkable advantages, with the main one of having high crop yields. In spite of the strain diversity, field trials were as high as 27-90 tons per ha of fresh tubers, representing 73-80 % of moisture, 15-17 % carbohydrates, about 2 % of protein and 0.1-1.8 % of fat (Kosaric *et al.*, 1984; Gibbons, 1989; Baldini *et al.*, 2004). Jerusalem artichoke can grow on marginal lands and in cold climate with poor fertilization requirements and a high resistance to wind, sand and biological diseases (Kosaric *et al.*, 1984). All these advantages make it a promising feedstock for many industrial applications.

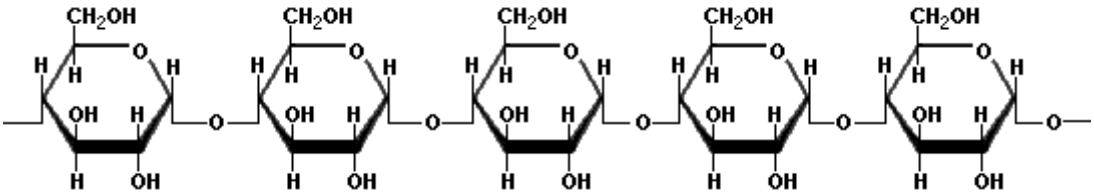
The Jerusalem artichoke has gained a considerable attention in recent years as raw material in various chemical and pharmaceutical industries. In renewable energy research field, great interest has specially focused on Jerusalem artichoke as a renewable source for bioethanol production. Sugars including sucrose, glucose and fructose are naturally present in this plant. The main polymeric form of carbohydrates found in this plant belongs to the fructan family, a polymer of fructose molecules, and represents from 7 % to 30 % of the fresh weight in the tubes, and from 9 % to 15 % of the fresh weight in stalks (Baldini *et al.*, 2004). Fructans with a short chain length are also known as fructooligosaccharides, whereas longer chains of fructans are named inulin. All fructans are linear poly fructose chains linked in ß(2-1) position with one terminal unit of glucose and other one of fructopyranose. The

average chain lengths of fructans range from 2 to 60 sugar units (Matias *et al.*, 2011). The inulin polymer presents several advantages in comparison to starch with, as an example, a faster dissolution in warm water and a lower viscosity at high concentration thanks to their chain structure (Figure I-8) which facilitates industry-scaling up operations (Chi *et al.*2011).

Jerusalem artichoke has been widely studied for bioethanol production by industrial yeasts. Indeed, yeasts naturally possess inulinase in cells and are able to ferment directly inulin to ethanol. The reported yields are up to 500 $L_{ethanol}.ton_{drymatter}^{-1}.ha^{-1}$ with *Saccharomyces cerevisiae*. In contrast, only few bacteria, mainly from *Bacillus*, *Clostridium*, and *Xanthomonas* genera were reported to exhibit inulinasic activities, a general requirement for inulin hydrolysis (Singh and Gill, 2006). Interestingly, an inulin-inducible inulinase activity was found in *Clostidium acetobutylicum* with pH and temperature optima of 5.5 and 47 °C, respectively (Kango and Jain, 2011). Up to now, only very few studies reported Jerusalem artichoke as substrate for hydrogen production by dark fermentation (Sciarria *et al*., 2010).



a. Inulin, as the main carbohydrate in Jerusalem artichoke plant, a fructose (n=approx.35) polymer branched in ß(2-1).



b. Amylose, as a starch component, a linear polysaccharide of glucose units branched in α-D-glucose

Figure I-8 Comparison of inulin and starch molecular structures.

In conclusion, crop residues, livestock, and food waste are potentially suitable substrates for hydrogen production by dark fermentation. Food waste gives the highest yield of hydrogen, followed by crop residues and animal manure. Nonetheless, for higher conversion efficiency, the agricultural waste generated by agricultural activities such as crop residues should be co-digested with animal manure using already existing biogas plants and

implementing a dedicated biohydrogen production stage. By coupling with methane bioprocesses, the treated effluent could finally be used as a fertilizer. In this scheme, the production of biohydrogen and biomethane might be used for heating and electricity generation or, in the case of biohydrogen, also as a chemical reactant. In particular, although only few studies have reported hydrogen production from Jerusalem artichoke plant and from inulin, its high carbohydrate content showed a promising potential for hydrogen evolution and constitutes and interesting model of study. Nevertheless, future research is still recommended to better understand the influence of feedstock composition and to optimise bioreactor performances and the co-digestion systems.

I.2 Dark fermentation process engineering with target of hydrogen production

The major limitation of biohydrogen production at an industrial scale concerns the low productivity and the low conversion yields of the fermentative biological processes. Based on current hydrogen productivity, industrial processes would require very large-volume reactors. Levin *et al.* (2004) reported that the minimum size of a bioreactor required to power a small proton exchange membrane fuel cell installation of 1 kW was 198 L, when considering H_2 productivity of 2.7 $L.L^{-1}.h^{-1}$ using dark fermentation and mesophilic conditions (Levin *et al.*, 2004). The productivity of hydrogen-producing bioreactors treating agri-waste is substantially lower than the result cited above because of the use of complex and polymeric organic substrates and also the mixed cultures as inoculum. However, the optimization of the operating conditions of biological reactors remains a key issue for the improvement of biohydrogen production. Specifically-optimized bioreactors could help to determine whether the use of agricultural waste in situ would be technically feasible and economically viable. To develop independent biohydrogen practical applications on farms, likely coupled with methane production, it is vital to consider concomitantly advances in biotechnology to enhance biohydrogen yield and biogas quality along with fuel cell development (Levin *et al.*,

2004). In order to meet these requirements, the following operating conditions must be considered.

I.2.1 Operation parameters

I.2.1.1 pH

pH is one of the most important factors to be regulated in dark fermentation processes (Chu *et al.*, 2008; Espinoza-Escalante *et al.*, 2009). Indeed, pH affects not only the yields of hydrogen production in mixed cultures, but can also modify by-product spectrum and impacts the global structure of microbial communities (Ginkel *et al.*, 2001; Temudo *et al.*, 2007; Ye *et al.*, 2007). Table I-3 summarizes the operating parameters in reactors treating agricultural residues inoculated with naturally mixed microbial cultures. Optimal H_2 production appears to take place with a pH of 5.0 - 6.0 for food waste (Kim *et al.*, 2004; Kim *et al.*, 2006; Doi *et al.*, 2009), whereas a neutral pH is recommended for crop residues and animal manure (Li *et al.*,2007; Yokoyama *et al.*, 2007a; Zhang *et al.*, 2007a; Kotsopoulos *et al.*, 2009). Two different types of experimentation have been performed to determine the optimal pH : one involved adjusting different initial pHs in a series of batch tests while the other maintained the same pH in continuous reactors during the fermentation process (Yokoyama *et al.*, 2007a; Karlsson *et al.*, 2008; Lee *et al.*, 2008). Li *et al.* (2007) investigated a large range of initial pH, from 4 to 8, in batch tests. They showed that a pH of 7-7.5 as optimal for the conversion of corn straw to biohydrogen (Li *et al.*, 2007). As the accumulation of by products, i.e. acetate and butyrate, lowered the pH of the medium, higher pH (i.e. around neutrality) led to better hydrogen yields. As suggested by Wang *et al.* (2009b), who reported that batch reactors with not regulated pH and treating sucrose are the systems most commonly studied, further investigations should focus rather on pH-controlled systems and on more complex organic wastes as substrates. In continuous reactors, in contrast, pH is usually controlled. A varied pH ranging from 4.5 to 6.5 was tested on tequila's vinasses in a semi-continuous CSTR reactor (Espinoza-Escalante *et al.*, 2009). It was concluded that a pH of 5.5 was optimal for hydrogen

production. A similar value was proposed in another study devoted to brewery waste in a CSTR with a pH ranging from 5.0-6.5 (Fan *et al.*, 2006a). As a general rule, the optimal pH in terms of biohydrogen production is within a range of 5.0-7.0 which probably favours the activity of the hydrogenases and is also suitable for microbial development in dark fermentation (Li and Fang, 2007).

In addition, the pattern of intermediate VFAs is different under variable pH conditions. Butyrate and acetate are the two main products, but at low pHs butyrate is preferentially produced. Hydrogen-producing butyrate-acetate pathways are favoured at pH 4.5-6.0 while at neutral or higher pH conditions, ethanol and propionate accumulate (Kim *et al.*, 2004; Hawkes *et al.*, 2007; Yokoyama *et al.*, 2007a; Pakarinen *et al.*, 2008). When using brewery waste as a substrate, Fan *et al.* (2006a) observed that, at pH 6.0 or below, acetate and butyrate were the major by-products whereas solventogenesis (acetone, butanol and ethanol) occurred at pHs higher than 6.5 (Fan *et al.*, 2006a). This was confirmed by Fang and Liu (2002) in a study investigating the effect of pH from 4.0-7.0 on by-product formation. At low pH, butyrate and acetate were dominant products while ethanol, lactate, propionate and caproate appeared at higher pH (Fang and Liu, 2002). Temudo *et al.* (2008) studied the impact of the pH on metabolic activity and microbial diversity in fermentation processes with glucose, xylose, and glycerol at 30 °C. They showed that a low pH conditions (<6), the product spectrum consisted mainly of butyrate and acetate while at high pH, the spectrum shifted to acetate and ethanol. It is noteworthy that under both high and low pH conditions, the fermentation pattern was clearly associated with the dominance of *Clostridium* species, whereas at intermediate pH, metabolic shifts involved higher microbial diversity (Temudo *et al.*, 2008). This suggests that pH effects result not only from a shift in metabolic pathways but also in major changes in microbial communities.

Table I-3: Optimal pH for biohydrogen production according to the category of organic substrate.

Substrate	Reactor	pH range	pH optimum	Reference
Corn straw	Batch	4-8 each0.5unit	7.0-7.5	Li *et al.*, 2007
Grass silage	Batch	4; 5; 6	6	Karlsson *et al.*, 2008
Rice bran	Batch	7initial	-	Noike and Mizuno, 2000
Wheat bran	Batch	7.0initial	-	Noike and Mizuno, 2000
Wheat straw	Batch	4-9	7	Fan *et al.*, 2006b
Cow waste slurry	Batch	6-7.5	7.0	Yokoyama *et al.*, 2007a
Cattle wastewater	Batch	4.5-7.5	5.5	Tang *et al.*, 2008
Foodwaste	Batch	6initial		Kim *et al.*, 2004
Foodwaste	CSTR	5.0-6.0	5.5	Shin and Youn, 2005
Foodwaste	ASBR	5.3 constant	-	Kim *et al.*, 2008
Foodwaste	CSTR	5.5-6.0 constant	-	Karlsson *et al.*, 2008
Foodwaste	CSTR	5.5 constant	-	Chu *et al.*, 2008
Vegetable kitchen waste	Batch	5.5-7 constant test	6.0-7.0	Lee *et al.*, 2008

I.2.1.2 **Biohydrogen Partial Pressure**

Many studies have already reported that partial pressure of hydrogen is a restrictive factor in the course of the fermentation of organic waste. The oxidation of reduced components such as Long-Chain Fatty Acids to VFAs, concomitantly with hydrogen production, is the consequence of a low biohydrogen concentration in the medium because reactions are thermodynamically unfavourable (Li, 2009). The positive Gibbs energy of LCFA degradation ($G^{\circ} = +48$ mJ.mol^{-1}) shows that the degradation of fat through the β-oxidation pathway is thermodynamically unfavourable and therefore requires an extremely low level of hydrogen partial pressure (see Equation I-13) (Li, 2009).

$$\text{n-LCFA} \rightarrow \text{(n-2) – LCFA} + 2\ \text{Acetate} + 2\ H_2 \quad \Delta G^{\circ} = +48\ \text{kJ.mol}^{-1} \qquad \text{(I-13)}$$

Additional formation of hydrogen could also derive from the degradation of acetate (see Equation I-14) (Angenent *et al.*, 2004). This conversion is thermodynamically unfavourable at

moderate temperatures and the reaction is therefore extremely sensitive to biohydrogen concentration. Furthermore, the inverse reaction, called homoacetogenesis, is rather favoured in the fermentation process and partly reduces the performance of bioreactors through the accumulation of acetate in the medium. By the increase of the hydrogen concentration in the medium due to microbial metabolism, not only biohydrogen production may be affected but also a shift of metabolic pathways towards solventogenesis has been observed, *i.e*. the accumulation of lactate, ethanol, acetone and butanol (Levin *et al.*, 2004). Recent research indicates, however, that the main factor leading to solventogenesis is the accumulation of volatile fatty acids rather than hydrogen partial pressure (Valdez-Vazquez *et al.*, 2006). Especially when feeding with a high glucose concentration, the intermediate acids produced, particularly butyric acid, initiate solventogenesis (Van Ginkel and Logan, 2005a).

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + CO_2 \quad \Delta G° = +104.6\ kJ.mol^{-1} \qquad \text{(I-14)}$$

To decrease pH_2 in the medium, especially in highly concentrated bioprocesses treating organic waste, agitation is the most usual technique. Chou *et al.* (2008) studied the conversion of brewery grains to hydrogen in a 100 L pilot bioreactor. Experiments showed that the rate as well as the yield of biohydrogen production increased from 1.8 $mL_{H2}.L_{reactor}^{-1}$ to 6.1 $mL_{H2}.L_{reactor}^{-1}$ while the stirring was speeded up from 20 to 100 rpm (Chou *et al.*, 2008). Several other alternatives exist to improve gas extraction, including gas sparing and biohydrogen stripping from reactor headspace by membrane absorption. Mizuno *et al.* (2000) showed that sparing nitrogen gas into a fermentor fed with simple sugars led to double the biohydrogen yield from 86.76 $mL_{H2}.g_{VS}^{-1}$ to 187.86 $mL_{H2}.g_{VS}^{-1}$. Other gases such as argon or a mixture of recirculation gases have also been used (Mizuno *et al.*, 2000; Van Groenestijn *et al.*, 2002). The main disadvantage of these techniques is that, regardless of the significant biohydrogen removal, the sparing gas dilutes the biohydrogen content and creates a further reduction in separation efficiency. In the event of up scaling to an industrial level, the high energy consumption in sparing processes and H_2 purification would raise the production costs, and the fluctuation in gas prices would impact directly on the economic viability of the

process. Membrane-absorption techniques offer other energy-effective alternatives for hydrogen removal from a gas mixture. Liang *et al.* (2002) reported a reduced biogas partial pressure by introducing a submerged hollow-fiber silicone membrane into the reactor. A Pd-Ag membrane reactor (Nielsen *et al.*, 2001) and a synthetic polyvinyltrimethyl silane membrane reactor (Teplyakov *et al.*, 2002) exhibited the highest hydrogen selectivity. The main disadvantage of using membrane-absorption techniques is the presence and the development of a biofilm over time which may favour the emergence of methanogenic bacteria.

Despite the different techniques available for reducing the partial hydrogen pressure, more research is still required to develop efficient and low cost gas purification systems aiming at the direct use of hydrogen from biogas to fuel cells at industrial scale.

I.2.1.3 **Temperature**

Temperature is often considered as one of the most important parameters affecting both biohydrogen production yields and microbial metabolisms in mixed cultures (Li *et al.*, 2007). Because of the complexity of the agri-waste and the variable operating conditions, no optimal temperature for hydrogen fermentation can be found from literature data. Most studies on fermentative hydrogen production have been carried out under mesophilic temperatures. Li *et al.* (2007) reported that 73 of 101 case studies were carried out at mesophilic temperatures. Crop residues usually present higher yields at thermophilic temperatures due to a better hydrolysis of the lignocellulosic compounds. For instance, the highest amounts of hydrogen from grass were obtained at 70 °C using a heat-treated inoculum from a dairy farm digester, *i.e.* 16 $mL_{H2}.g_{VS}^{-1}$ (Pakarinen *et al.*, 2008). Regarding food waste, thermophilic temperatures seem more suitable to hydrogen production despite significantly different observations reported in the literature. These differences might be due to the origin of the inoculum, the quantity of readily-biodegradable compounds as well as the operating conditions. At 55 °C, acetate was the dominant by-product while a propionate production pathway was favoured at

20 °C (Karlsson *et al.*, 2008). To examine the effect of the fermentation temperature on biohydrogen production, dairy cow waste slurry was cultured at 37 °C, 50 °C, 55 °C, 60 °C, 67 °C, 75 °C and 85 °C (Yokoyama *et al.*, 2007b). Although two optima of production were observed at 60 °C and 75 °C, with yields of 29.25 $mL_{H2}.g_{VS}^{-1}$ and 18.5 $mL_{H2}.g_{VS}^{-1}$, the increase in hydrogen production globally correlated with higher operating temperatures. Performances were also influenced by changes in the microbial community structure. The structure of the microflora was significantly different at the two optimal fermentation temperatures. At 60 °C, the predominant bacteria were affiliated to *Bacteroides xylanolyticus*, *Clostridium stercorarium*, and *Clostridium thermocellum*, while at 75 °C three strains of the extremophilic thermophilic bacterium *Caldanaerobacter subterraneus* were dominant (Yokoyama *et al.*, 2007b). Without pretreatment of the initial inoculum, temperatures higher than 60 °C are recommended in order to reduce hydrogen-consuming activity (Yokoyama *et al.*, 2007b). In any event, the main disadvantage of thermophilic anaerobic fermentation processes is the energy requirement for heating and maintenance.

I.2.1.4 **Hydraulic Retention Time**

The hydraulic retention time (HRT) is defined as the average time for a given flow to pass through a reactor, and consequently, HRT represents the time that an inert particle remains in a reactor operated in continuous mode (CSTR). It is calculated by dividing the volume of the reactor by the influent flow rate. Many studies have investigated the impact of this operation parameter on bioH_2 production. The optimal HRT in fermentative hydrogen production system varied greatly depending on the type of reactors, the pH level and the microbial community (Hawkes *et al.*, 2007; Wang *et al.*, 2009a). In CSTR, the microbial biomass is well mixed with high-speed agitation, and non-growing bacteria are rapidly washed out from the reactor. In this case, the biomass growth rate (μ) directly corresponds to the inverse of HRT, so called the dilution rate (D). The biomass concentration is therefore linked to HRT which, in counterpart, influences the biohydrogen yields and productivities. From this, HRT in CSTR is desired to be longer than in reactors capable to maintain more

biomass *e.g.* immobilized-cell reactors (Table I-4). However, in terms of hydrogen production, short HRTs are recommended to avoid the presence of microorganisms with lower growth rate such as methanogens (Hawkes *et al.*, 2007). Thus, considering this, the optimal HRT values depend mainly on the organic substrate and the microbial community. As an example, Fang and Liu (2002) observed an occurrence of methane production in a CSTR even at a short HRT of 6 h (Fang and Liu, 2002). In this study, the seed sludge corresponded to the outlet of an anaerobic digester with no pretreatment, and therefore methanogens implemented even in short HRT. In contrast, another study from Fan *et al.* (2006b) did not detect any methane accumulation on sucrose during 10 months of operation on brewery waste, in a CSTR seeded with a heat-treated inoculum and operated at 18 h HRT (Fan *et al.*,2006c).

Table I-4: Summary of optimal HRT associated to the reactor type reported in the literature (adapted and completed from Wang *et al*., 2009b).

substrate	Reactor type	Optimal HRT (hours)	Hydrogen yield	References
Glucose	CSTR	0.5	1.81 $mol_{H2}.mol_{glucose}^{-1}$	Zhang *et al.*,2007
Glucose	CSTR	10	1.95 $mol_{H2}.mol_{glucose}^{-1}$	Zhang *et al.*, 2006
Glucose	CSTR	10	1.63 $mol_{H2}.mol_{glucose}^{-1}$	Wu *et al.*, 2008a
Sucrose	CSTR	4	4.7 $mol_{H2}.mol_{sucrose}^{-1}$	Chen *et al.*, 2008b
Sucrose	CSTR	8	4.52 $mol_{H2}.mol_{sucrose}^{-1}$	Chen and Lin, 2003
Fructose	CSTR	8	1.68 $mol_{H2}.mol_{hexose}^{-1}$	Lee *et al.*, 2007
Xylose	CSTR	12	1.63 $mol_{H2}.mol_{xylose}^{-1}$	Wu *et al.*, 2008 b
Starch	CSTR	12	1.5 $mol_{H2}.mol_{hexose}^{-1}$	Lin *et al.*, 2008
Starch	CSTR	12	0.92 $mol_{H2}.mol_{glucose}^{-1}$	Arooj *et al.*, 2008
Sucrose	UASB	8	1.5 $mol_{H2}.mol_{sucrose}^{-1}$	Chang and Lin, 2004
Sucrose	UASB	8	3.6 $mol_{H2}.mol_{sucrose}^{-1}$	Chang and Lin, 2006
Glucose	Anaerobic biofilm fluidized bed reactors	0.25	1.7 $mol_{H2}.mol_{glucose}^{-1}$	Zhang *et al.*, 2008
Sucrose	Packed-bed bioreactor	4	3.9 $mol_{H2}.mol_{sucrose}^{-1}$	Lee *et al.*, 2003
Xylose	Immobilized-cell continuously stirred anaerobic reactor	6	0.8 $mol_{H2}.mol_{xylose}^{-1}$	Wu *et al.*, 2008b

I.2.1.5 **Organic loading rate (OLR)**

The organic loading rate is also an important parameter of continuous hydrogen fermentative systems and corresponds to the quantity of organic compounds introduced per

day and per volume unit of reactor. Considering the energy input of a continuous fermentation system, higher OLR are recommended to gain in operation cost. However, high OLRs seem to unfavour hydrogen production. Indeed, for increasing OLR, fermentative products such as VFAs accumulate, and the microbial metabolism can switch from acidogenesis to solventogenesis (Lee *et al.*, 2008) and may be even accompanied by sporulation during this stage (DÜrre *et al.*, 2002).

Furthermore, Kyazze *et al.* (2006) showed that an increasing loading rate can led to a decrease in hydrogen yield. In this study, CSTRs were fed with sucrose and seeded by heated anaerobic digester sludge. The hydrogen yield was reduced from 1.7 $mol_{H2}.mol_{hexose}^{-1}$ to 0.8 $mol_{H2}.mol_{hexose}^{-1}$ at 10 $g.L^{-1}.d^{-1}$ and 50 $g.L^{-1}.d^{-1}$, respectively. The butyrate pathway of biohydrogen production was the most impacted but the molecular mechanism of inhibition was not clearly established (Kyazze *et al.*, 2006). Another experiment carried out in ASBR (anaerobic sequential batch reactor) investigated the effect of OLR ranging from 25 to 40 $g.L^{-1}.d^{-1}$ of sweet sorghum juice on the hydrogen production yields (Saraphirom and Reungsang, 2010). The highest hydrogen yield of 0.53 $mol_{H2}.mol_{hexose}^{-1}$ and maximum production rate of 413 $mL.L^{-1}.d^{-1}$ were observed at an OLR of 30 $g.L^{-1}.d^{-1}$. Based on microbial analysis, the presence of *Lactococcus lactis* and *Lactobacillus sp.* were suggested to cause low hydrogen yields through lactate accumulation. But the presence of lactic acid bacteria could not explain the low hydrogen yields observed at the highest OLR of 35 or 40 $g.L^{-1}.d^{-1}$, since the bands affiliated to *Lactobacillus sp.* disappeared, suggesting that solventogenesis could have occurred (Saraphirom and Reungsang, 2010). Ozmihci and Kargi (2011) reported an optimal OLR at 1.38 $g_{TS}.L^{-1}.d^{-1}$ for wheat starch solution at 10 $g.L^{-1}$ to reach a high hydrogen yield of 109 $mL.g_{TS}^{-1}$. In this study, pure cultures of *Clostridium butyricum* and *C. pasteurianum* were used as inocula and no hydrogen competitors or consumers were considered. The decrease in hydrogen yield at higher OLR was probably due to the metabolic changes occurring through VFA accumulation (Ozmihci and Kargi, 2011). Overall, there has not been found a clear evidence of the effect of OLR on hydrogen

production pathways in mixed culture where cellular mechanisms as well as microbial interactions are involved, and further investigations are still required in this field.

I.2.2 **Bioreactor configuration**

At laboratory-scale, most studies dealing with dark fermentation from solid substrates have been performed in batch reactors (Fan *et al.*, 2006b; Pakarinen *et al.*, 2008). Batch-mode reactors possess the advantage of being easily operated and flexible. This has resulted in the wide utilization of batch reactors for determining the biohydrogen potential of organic substrates. However, in an industrial context, for practical reasons of waste stock management and for economic considerations, continuous bioprocesses are recommended. To date, no biohydrogen industrial-scale reactor has been set up, but it is expected that bioreactor design and system configuration will be similar to methane biogas plants: only the operational parameters may vary between these two anaerobic applications. In view of the extensive the experience acquired in biogas plants treating agricultural organic waste, especially in Germany, the most probable reactor for biohydrogen production would be a vertical, continuously-stirred tank reactor with different types of mixers (Weiland, 2006). More than half of this type of reactor is covered with a single or double-membrane roof to store the biogas (Figure I-9) (Weiland, 2006). Within the one-stage fermentation concept at laboratory-scale, continuous stirred tank reactors (CSTR) are the most common continuous system used for anaerobic digestion (Lay, 2001; Kotsopoulos *et al.*, 2009) in hydrogen production research on substrates such as pig slurry (Kotsopoulos *et al.*, 2009), swine manure (Zhu *et al.*, 2009) for food waste (Shin and Youn, 2005; Wang *et al.*, 2009b) (Table I-2). Other studies have reported successful use of ASBR, rather than CSTR, for food waste conversion (Kim *et al.*, 2008). Only few studies have concerned the processes for treating high-solid-content agricultural waste (Li *et al.*, 2007). The reasons could be the instability of such systems in the course of hydrogen fermentation due to the highly variable composition of the feed and the metabolic instability of the microbial consortia. A remarkable reactor design was set up by Jayalakshmi *et al.* (2009) to investigate kitchen waste in hydrogen

conversion. This was a pilot-scale, inclined, plug-flow reactor, cylindrical in shape and kept at a 20° angle to the horizontal to facilitate movement of the waste. A screw arrangement inside the reactor, serving to push the material from the inlet at the bottom to the outlet at the top was designed with 14 leads to maintain seven days retention time, which was important for the solid waste to have sufficient hydrolysis time (Jayalakshmi *et al.*, 2009). Additionally, a start-up in batch mode favoured the formation of stable microflora granules, and consequently enhanced seed source activity (Chou *et al.*, 2008; Jayalakshmi *et al.*, 2009).

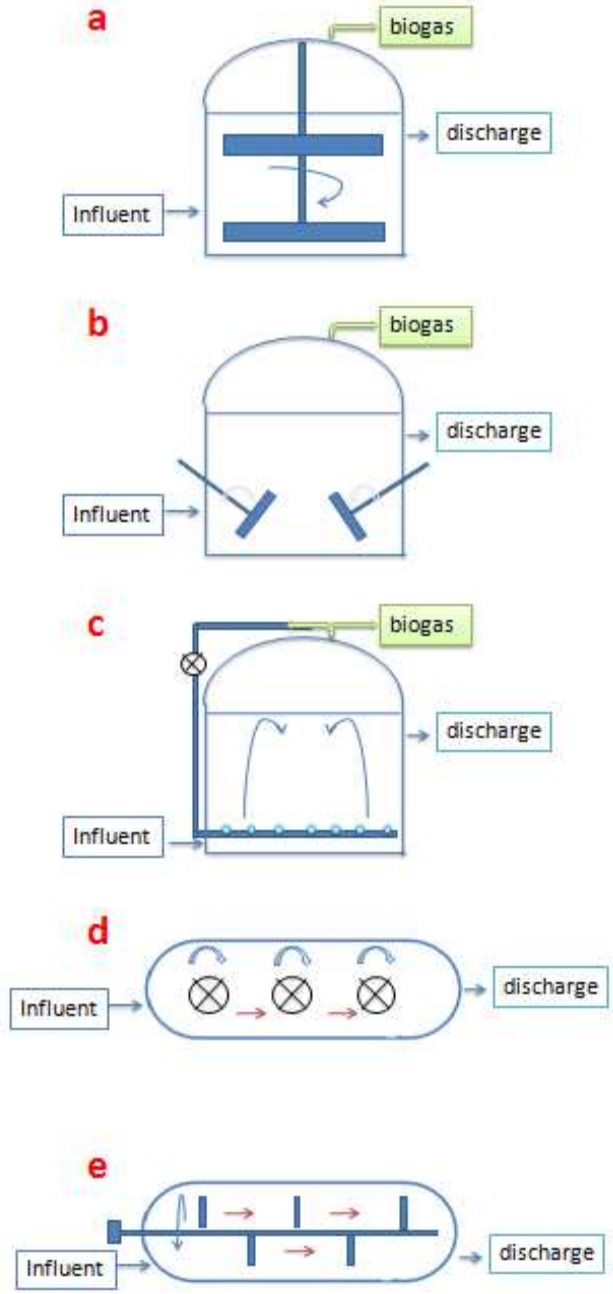


Figure I-9: Different types of anaerobic digestion plant, adapted from Weiland 2006.

a/b/c: vertical, completely-stirred tank reactor (a/b: mechanical stirring; c: biogas mixing), d/e: Horizontal plug-flow reactor (mechanical stirring).

I.2.2.1 The dark fermentation processes within the biorefinery concept

Hydrogen production by biotechnological ways is completely included in the biorefinery concept. As defined by the international energy agency, a biorefinery is a sustainable processing which converts biomass, including a mixture of organic materials, to multi-products: bio-base products (food, chemical molecule, materials) and bioenergy (biofuel, heat or/and power). It can take advantage of different biomass constituents and intermediates to maximize the value derived from feedstock. In dark fermentation processes, the first hydrolysis steps aim to decompose organic polymers, or a mixture of them, such as carbohydrates and proteins, to soluble molecular components such as oligosaccharides and amino acids, and make them available for acidogenic bacteria. In the subsequent step, acidogenic bacteria convert sugars and amino acids to metabolic end-products such as VFAs, alcohols and CO_2 and H_2. Acetogenic bacteria can convert all VFAs and alcohols to acetic acid, at low hydrogen partial pressure ($<10^{-3}$ atm), and therefore only in symbiotic relationship with methanogens. Moreover, homoacetogenic bacteria can directly convert CO_2 and H_2 to acetate. Therefore, besides hydrogen, VFAs, alcohols and organic acids are rich in energy but not used by other fermentative microorganisms, except in presence of methanogens, exclusively due to thermodynamical considerations. In a biorefinery concept, several other processes should be addressed afterwards to valorise these end-products. Thus the hydrogen produced in dark fermentation stage becomes part of a process stream.

I.2.2.2 Coupling dark fermentation to anaerobic digestion (methane production)

Two-stage systems coupling of hydrogen fermentation with methane production has been widely investigated for treating substrates such as livestock waste and food waste (Koutrouli *et al.*, 2009; Venetsaneas *et al.*, 2009; Wang *et al.*, 2009b). Such a two-phase anaerobic digestion system was first proposed by Pohland and Ghosh (1971). In this system, only fast-growing acidogenic bacteria are dominant in the first step and produce VFAs, whereas slow-growing acetogens and methanogens are the main microorganisms present in the second

step in which VFAs are converted to methane and carbon dioxide. This combination of such fermentation systems greatly enhances the energy conversion compared to the one-stage process. Liu *et al.* (2006) observed an increase of 21 % in methane yield, *i.e.* 500$mL_{CH4}.g_{VS}^{-1}$ with an associated hydrogen yield of 47 $mL_{H2}.g_{VS}^{-1}$, from household waste, in a two stage process compared to one methanogenic step. Chu *et al.* (2008) also reported a successful association of reactors for hydrogen and methane production from the organic fraction of municipal solid waste (OFMSW), under ph-controlled conditions of fermentation for each step: respectively, 55 °C, pH 5.5, 31 h HRT and 35 °C, neutral pH, 120 h HRT. They demonstrated that a short HRT and acid pH prevent methanogenic activity in the acidogenic stage. After optimization of the reactor association system, high biogas yield and CH_4 content, *i.e.* 464 $ml_{CH4}.g_{VS}^{-1}$, 70 %-80 % respectively, were observed thanks to the hydrolytic activity in the first step. The treatment time was also reduced: an HRT of 5 days was already enough for the methane stage instead of a more usual minimal HRT of 10 - 15 days in thermophilic and mesophilic conditions, respectively (Li *et al.*, 1999). Another study estimated that 5.78 % of the influent COD was converted to hydrogen in the first stage, and 82.18 % of COD converted to methane in the second stage, with an overall COD removal efficiency improved (Wang and Zhao, 2009). In this study, high hydrogen yield of 65 $mL_{H2}.g_{VS}^{-1}$ and methane yield of 546 $mL_{CH4}.g_{VS}^{-1}$ from food waste were observed using an inoculum derived from the indigenous microbial cultures contained in this substrate (Wang and Zhao, 2009).

I.2.2.3 **Coupling dark fermentation to photofermentation**

Another suggested two-stage system consists of the combination of dark and photo-fermentation. Nath *et al.* (2008) described a process associating dark and photo-fermentation in a sequential batch reactor. A glucose-based media was inoculated with *Enterobacter cloacae* DM11 to produce H_2, CO_2 and VFAs in dark fermentation. Then, in a second reactor, acetate was subsequently used by *Rhodobacter sphaeroides* O.U.001 to form hydrogen. The hydrogen yield in the first stage was about 3.31 $mol_{H2}.mol_{glucose}^{-1}$ and in the second stage in a range of 1.5-1.72 $mol_{H2}.mol_{acetic\ acid}^{-1}$, equivalent to 3-3.4 $mol_{H2}.mol_{glucose}^{-1}$.

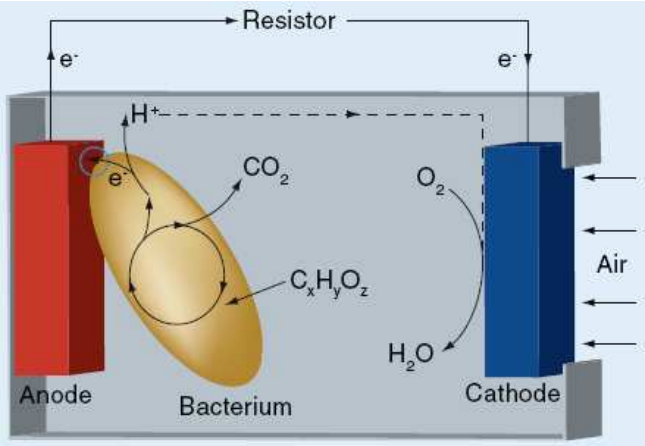
Thus, the overall yield exceeded 6 $mol_{H2}.mol_{glucose}^{-1}$, which is higher than of the maximum 4 $mol_{H2}.mol_{glucose}^{-1}$ obtained with the dark fermentation process alone. The use of agri-waste as a substrate in these types of association remains to be tested.

I.2.2.4 **Coupling dark fermentation to microbial electrochemical cells**

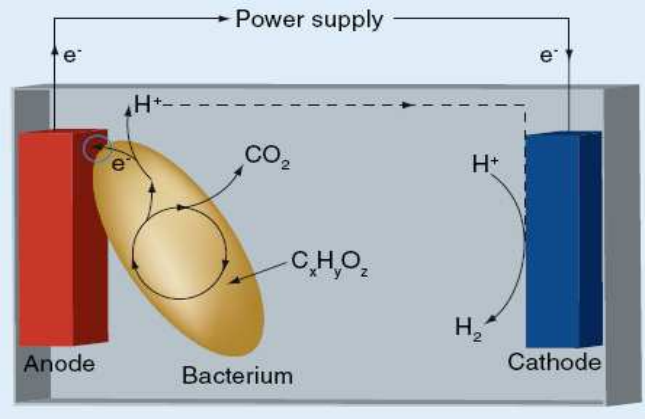
Microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) are biological reactors which can transform the chemical energy of the organic matter into electricity or hydrogen through direct electron exchange. In such systems, acetate and butyrate can be utilized to produce electricity in MFCs (Liu *et al.*, 2005). The schematic representation of a basic microbial fuel cell is presented in Figure I-10a. The bacteria can grow on the anode from various carbon sources such as simple sugars or VFAs issued from the first dark fermentation process. The electrons are then transferred to the anode and the protons are migrating into the medium solution. At the cathode side, the protons react with dissolved oxygen to form water, meanwhile the electrons are transferred to provide electricity current. When no oxygen is provided at the cathode, hydrogen can be produced in bio-electrolysis cell, so-called Microbial Electrolysis Cell (MEC). This system needs an external power supply (~0.2V) to increase the electronic potential for reducing the protons and to form hydrogen from water splitting at the cathode (Figure I-10b). This microbial electrolysis cell required 10-fold less of power by comparison with classic water electrolyser (2V), and therefore, it provides hydrogen at a lower energy cost (Liu *et al*., 2010).

These techniques are very active at a research stage, and are very attractive to be coupled to dark fermentation processes and to enhance the overall hydrogen production yield. Lu *et al.* (2009) reported the combination of dark fermentation in CSTR from molasses and a MEC, with a high hydrogen production rate of 2.11 $L.L^{-1}.d^{-1}$. However, with time, the process failed to hydrogen production since methanogens implemented in the biofilm on the electrode surface. Another investigation made by Lalaurette *et al.* (2009) showed up to 980$mL_{H2}.g_{COD}^{-1}$ generated in a two stage dark-fermentation and MEC system, with about 800mL $mL_{H2}.g_{COD}^{-1}$ from the MEC alone, on cellobiose. But the accumulation of CH_4 was also observed over

experimental time. The authors stated that long cycle of batch times (3-4 days) seemed to favour the growth of methanogens, and suggested to control the cycle duration. Very recently, Tommasi *et al.* (2012) operated successfully a MEC to generate hydrogen from sodium acetate as artificial effluent. A high yield of 2.41 $mol_{H2}.mol_{sodium\ acetate}^{-1}$ was reached (Tommasi *et al.*, 2012). However, the effluents coming from dark fermentation processes are mostly acid (pH 5 – 6) and should be increased to neutrality for being used in the following MEC system, which constitutes a major drawback for industrial purpose (Gomez *et al.*, 2011). Further research efforts may therefore concentrate on improving the efficiency and stability of MECs when outlet of fermentation reactors is directly introduced into the anodic compartment (Logan *et al.*, 2006).



a. Single-chamber microbial fuel cell (MFC) generating electricity with bacteria-formed biofilm at the anode, and air introduced at the cathode.



b. Single-chamber microbial electrolysis cell (MEC) forming hydrogen with external energy supply to drive the electron flows from the anode where the bacterial biofilm oxidizes the organic matter to the cathode where electrons are accepted to generate hydrogen gas.

Figure I-10: Schematic representations of (a) microbial fuel cells (MFCs) and (b) microbial electrochemical cells (MECs) (from Liu *et al*., (2010)).

I.3 Microbiology fundamentals of biohydrogen production from agricultural waste

In a previous review paper, Nandi and Sengupta (Nandi *et al.*, 1998) listed the major hydrogen-producing bacteria related to strict anaerobic genera (clostridia, methylotrophs, rumen bacteria, methanogenic bacteria, archaea), to facultative anaerobic genera (*Escherichia coli*, *Enterobacter*, *Citrobacter*) and to aerobic genera (*Alcaligenes*, *Bacillus*). In relation to biohydrogen production from agricultural waste, *i.e.* in mixed cultures, three classes of microorganisms can be distinguished: hydrogen producers, hydrogen consumers and metabolic competitors.

I.3.1 The biohydrogen producers

Although pure cultures have been intensively investigated over the past years, involving amongst of others *Bacillus coagulans* (Kotay and Das, 2007), *Thermoanaerobacterium* sp. (O-Thong *et al.*, 2008b), *Enterobacter aerogenes* (Fabiano and Perego, 2002), *Clostridium butyricum* (Chen *et al.*, 2005), only few studies refer to the characterization of mixed cultures. A large range of microbial sources has been used to obtain inocula for biohydrogen production, including anaerobic sludge from municipal wastewater plants and cow dung composts (Chu *et al.*, 2008; O-Thong *et al.*, 2008c; Tang *et al.*, 2008; Wang *et al.*, 2009b), cattle or dairy residue composts (Fan *et al.*, 2004; Fan *et al.*, 2006b), sludge from palm oil mill effluent (Vijayaraghavan *et al.*, 2006a; Chong *et al.*, 2009), soil, rice straw compost, fermented soy bean meal (Noike and Mizuno, 2000) as well as landfill lixiviates (Karlsson *et al.*, 2008; Li *et al.*, 2008). Akutsu *et al.* (2008) showed that the origin of the inoculum affects the overall performance of the bioreactor. In another study, four natural mixed-microflora seed sources (sludge from sewage treatment; cow dung compost; chicken manure compost; and river sludge) were tested for fermentation in a

hydrogen reactor treating cattle wastewater, and sewage sludge showed the highest hydrogen-producing potential (Tang *et al.*, 2008).

Another investigation on the effect of the inoculum source on grass silage fermentation, *i.e*. sludge from a dairy farm digester and from a wastewater treatment plant, showed only significant biohydrogen production for bioreactors inoculated with the dairy farm digester sludge (Pakarinen *et al.*, 2008). This suggests that acclimation of the seed source is a major parameter that needs to be taken into account for biohydrogen fermentation.

From hydrogen-producing mixed cultures, a wide range of species have been isolated, more specifically from the genera *Clostridium* (*Clos. pasteurianum*, *Clos. saccharobutylicum*, *Clos. butyricum*), *Enterobacter (Ent. aerogenes)* and *Bacillus* under mesophilic conditions; and from the genera *Thermoanaerobacterium* (*Thermoanaerobacterium thermosacchatolyticum) Caldicellulosiruptor (C. saccharolyticus), Clostridium thermocellum, Bacillus thermozeamaize* under thermophilic or extremophilic temperatures (Hawkes *et al.*, 2002; Chang *et al.*, 2008b; O-Thong *et al.*, 2008b; Ivanova *et al.*, 2009; Karakashev *et al.*, 2009). Under mesophilic conditions, mainly sporulating bacteria of the *Clostridium* genus were found in mixed mixtures, likely because of a systematic use of heat shock pretreatment of the inoculum. In thermophilic conditions, *Thermoanaerobacterium sp.* is preferentially selected by the operating conditions in mixed cultures (O-Thong *et al.*, 2008b).

As to microbial performances, a biohydrogen yield of 3.8 $mol_{H2}.mol_{glucose}^{-1}$ at 70 °C which is very close to the theoretical maximum, was reported for *Caldicellulosiruptor saccharolyticus* (Ivanova *et al.*, 2009). Maximum hydrogen production of 2.53 $mol_{H2}.mol_{hexose}^{-1}$ was observed for *Thermoanaerobacterium thermosaccharolyticum* at a temperature of 60 °C (O-Thong *et al.*, 2008c). Other thermophilic hydrogen producers reached maximum hydrogen yields ranging from 1.5 to 3.3 $mol_{H2}.mol_{hexose}^{-1}$ for *Thermotoga elfii, Caldicellulosituptor saccharolyticus, Clostridium thermocellum, Clostridium*

thermolacticum. Clostridium thermobutyricum, and Clostridium thermosaccharolyticum (Wiegel *et al.*, 1989 ; Vancanneyt *et al.*, 1990 ; de Vrije *et al.*, 2002; van Niel *et al.*, 2002; Collet *et al.*, 2004; Levin *et al.*, 2006). Higher conversion yields were observed at high temperature for such microbes. This may partly explain the higher performances observed in bioreactors treating organic waste as well as the fact that hydrolysis is favoured at thermophilic temperatures.

I.3.2 H_2 consumers and metabolic competitors

Three groups of bacteria are known to interfere directly or indirectly, by diversion of the biohydrogen potential from carbohydrates, *i.e*. the Sulfate-reducing bacteria (SRB), the Methane-producing Bacteria (MPB), and the Homoacetogenic Bacteria (HAB) (see

Figure I-5, page 49).

I.3.2.1 Homoacetogenic bacteria

Homoacetogenic bacteria are strictly anaerobic microorganisms which catalyse the formation of acetate from H_2 and CO_2. They were first observed by Fischer *et al.* (1932). *Clostridium aceticum* and *Clostridium thermoaceticum* were the model species used to elucidate the metabolic pathway (Wieringa, 1939; Fontaine *et al.*, 1942). They possess special enzymes which catalyse the formation of acetyl-CoA that is converted either to acetate in catabolism or to cell carbon in anabolism. The homoacetogens are very versatile anaerobes, which convert a variety of different substrates to acetate as the major end product (Diekert, 1994). This implies, therefore, that in experimental studies the biohydrogen production measured might be lower than the expected value calculated from the accumulation of acetate (Antonopoulou *et al.*, 2008). Kotsopoulos *et al.* (2009) used pig slurry as substrate in a CSTR and observed that the actual production of hydrogen was substantially lower than the value expected from VFA accumulation. As no methane was detected in the biogas and the propionate mass balance did not explain hydrogen losses,

hydrogen was assumed to be consumed by acetogenic bacteria (Kotsopoulos *et al.*, 2009). Siriwongrungson *et al.* (2007) reported that considerable homoacetogenesis occurred in CSTR reactors using digested dairy manure as inoculum and operated under thermophilic temperatures (Siriwongrungson *et al.*, 2007). It was shown that the biohydrogen produced from butyrate oxidation reacted rapidly with CO_2 to form acetate by homoacetogenesis. Unfortunately, the pretreatment of the inoculum by heating to select spore-forming bacteria is not suitable for inhibiting of homoacetogenic bacteria since some of them belong to the same genus *Clostridium* (Oh *et al.*, 2003). Thus, only operating parameters could favour biohydrogen production, *e.g.* by removing CO_2 from the headspace (Park *et al.*, 2005).

I.3.2.2 **Sulfate-Reducing Bacteria**

According to theoretical thermodynamics, the most efficient biochemical reaction using hydrogen involves the sulfate/nitrate-reducing microorganisms ($\Delta G° = -165$ kJ.mol^{-1}), even at a low hydrogen concentration of only 0.02 ppm in the presence of sulfate or nitrate (Cord-Ruwisch *et al.*, 1988). It has been shown that SRB have a thermodynamic advantage over MPB and HAB (Valdez-Vazquez *et al.*, 2009). Some waste especially from pulp/paper industry, sea-food processing, distilleries, edible oil and wet corn milling, contains high sulfate concentrations which perturb hydrogen anaerobic digestion as well as produce hydrogen sulfide gas which is hazardous for fuel cells (Lin and Chen, 2006; Briones *et al*, 2009). Short HRTs are not sufficient to inhibit these microorganisms. Even at a HRT of 2h, the interspecies transfer metabolites such as hydrogen, carbon dioxide and VFA, are immediately consumed by SRB under sulfate-rich conditions (Valdez-Vazquez *et al.*, 2009). At longer HRT, hydrogen is converted either to methane with carbon dioxide by MPB under sulfate-limited conditions (Mizuno *et al.*, 1998). Along with the concentration of sulfate and HRT, pH is a key factor in sulfate reduction. Indeed, pH values lower than 6 significantly inhibit the activity of SRB (Mizuno *et al.*, 1998; Lin and Chen, 2006).

I.3.2.3 **Methanogens**

Methanogens are considered as the main hydrogen-consuming microorganisms in anaerobic environments (Morvan *et al.*, 1996; Sterling *et al.*, 2001; Weijma *et al.*, 2002). Many options exist for inhibiting methanogenesis: chemical inhibition, low pH control, heat treatment of the inoculum, short hydraulic retention times.

The most commonly used chemical inhibitors are Bromoethanesulfonate (BES), acetylene and chloroform (Li *et al.*, 2007). BES is specific against methanogens and acts as an analog of the coenzyme M in the respiratory chain. However, treating with effective concentrations of BES is not environmentally friendly and too costly for large-scale operations (Li *et al.*, 2007). pH is also a factor in preventing methanogenic activity since most methanogens can only grow at a narrow pH range from 6 to 8 (Chen *et al.*, 2002). In absence of pH control during a batch process, an acidic initial pH is strongly recommended (Bainotti and Nishio, 2000; Lu *et al.*, 2009). The most common treatment of inoculum is heating the medium to around 100 degrees for approximately ten minutes to select spore-forming, hydrogen-producing bacteria. Methanogens do not sporulate and do not survive under such conditions (Lay *et al.*, 2005; Vijayaraghavan *et al.*, 2006b). Because methanogens present low growth rates (approx. 0.2 h^{-1}), the application of short HRT (< 8 h) quickly leads to a washout of methanogens from the reactor, when no biofilm is formed. To obtain stable hydrogen production in a methane-free biogas, the optimal HRT observed were 3-6 h, 9h, 18h up to 48h for respectively, molasses, bean curd waste, brewery waste and food waste (Shin and Youn, 2005; Fan *et al.*, 2006a; Aceves-Lara *et al.*, 2008; Chang *et al.*, 2008a). In a kinetic study of hydrogen production in an anaerobic system, Chen *et al.* (2006) calculated a maximum specific growth rate for methanogenic microflora of 0.172 h^{-1}. They concluded that HRT of less than 6h are highly recommended to wash out selectively the methanogens in continuous reactors (Chen *et al.*, 2006; Valdez-Vazquez *et al.*, 2009).

I.3.2.4 **Lactic Acid Bacteria**

Noike *et al.* (2002) studied the inhibition of hydrogen production by lactic acid bacteria (LAB). They observed the replacement of hydrogen fermentation by lactic acid fermentation when two lactic acid bacteria (LAB) strains, i.e. *Lactobacillus paracasei* and *Enterococcus durans*, were cultivated with two hydrogen-producing strains, *Clostridium acetobutylicum* and *Clostridium butyricum*. Secretion of bacteriocins was recognized as the inhibitory effect and temperatures above 50°C were proposed to prevent LAB influence (Noike *et al.*, 2002). In mesophilic systems, LAB growth could not be limited by temperature, and the accumulation of lactic acid led to the instability of the mixed culture processes. Indeed, Wang *et al.* (2009b) showed that lactic acid inhibited hydrogen fermentation in a two-stage continuous system using food waste as substrate. The hydrogen yield dropped from 71 to 49 $mL_{H2}.g_{VS}^{-1}$ when the lactic acid increased from 2.3 to 4.4 $g.L^{-1}$. Increasing the organic loading rate resulted in an increase in lactic acid concentration and in the microflora indigenous in food waste, *i.e*. lactic acid bacteria, and then led to the perturbation of the system if no pretreatment had been previously carried out (Wang *et al.*, 2009b).

I.4 **Conclusion**

The present literature overview reports recent findings on biohydrogen production from agricultural waste by dark fermentation. Three categories of agricultural residue have been considered in this chapter: (i) the waste directly generated from agricultural production (ii) animal manure and (iii) food waste. It is shown that all three possess great potential as substrate for hydrogen production by dark fermentation, in decreasing order: food waste, crop residues and livestock waste. Nevertheless, further research is still necessary to better understand the impact of the composition of the substrate on biohydrogen performances. Moreover, the biological processes involved are not only influenced by the composition of the organic waste, but also they are also highly dependent of the operating conditions. Key

operational parameters such as low pH (approx. 5.5), low partial pressure (< 10^{-3} atm), high temperature (>55°C) as well as acclimated microbial communities are recommended. These operating parameters affect not only the yields of biohydrogen in mixed culture, but also redirect the end-product pattern spectrum and impact the structure of the microbial communities. Finally, it is important to distinguish three classes of microorganisms that require further characterization in mixed cultures: hydrogen producers, hydrogen consumers and metabolic competitors. The limitation of the presence of various hydrogen consumers and the control of the occurrence of H_2 consuming pathways in mixed cultures constitute the main challenge for further improving the stability of bioreactors treating agricultural waste.

CHAPTER II. MATERIALS AND METHODS

TABLE OF CONTENTS

II.1 Organic substrate sources and preparation

II.1.1 Substrates used in the Biochemical Hydrogen Potential (BHP) tests

Twenty-six solid organic substrates were collected for their evaluation in biochemical hydrogen potential (BHP) test experiments. They were gathered into four groups according to their main composition : (i) the first group corresponded to substrates rich in carbohydrates, including apples (royal gala), carrots, Jerusalem artichoke roots, maize flour, oats, potato, wheat flour; (ii) the second group corresponded to substrates rich in proteins with soybean milk cake, chicken meat, cow manure with straw, fish residues, and meat waste from restaurant, (iii) the third group corresponded to agri-industrial co-products, with food waste from restaurant, rapeseed oil cakes, sunflower oil cakes, grape marc, vegetable waste from restaurant, fruit peels (orange peels and banana peels) and maize cob, and (iv) the last group corresponded to crop residues including Jerusalem artichoke leaves and stalks, giant reed stalks and leaves, maize stalks, rice straw and sorghum stalk.

Apples, carrots and potatoes were originated from commercial market, and the whole fruit including peel and grain were used. Oat and wheat flour corresponded to commercial products rich in starch but with small amounts of fiber content. Jerusalem artichoke roots and stalk were harvested in a local farming in September during the flowering time. The crop residues were originally collected during the harvest season, and were dried naturally in air on farms. Food waste was collected in a food restaurant over one month. Within food waste, meat waste and vegetable waste were manually separated. All the collected food waste were then stored at -20 °C. Fruit peels were collected at a food restaurant and stored at -20 °C. Rapeseed oil cakes, sunflower oil cakes, grape marc, fish residues were collected from manufacturing industries. Cow manure with straw was collected from a farm.

All the substrates were milled in a blender SM 100 and sieved with a mesh at 3 mm, after freeze-drying of 48 hours (HetopowerDry PL 3000, ThermoElectron Corporation).

II.1.2 Preparation of substrate feeding solution for continuous hydrogen production in sCSTR

As discussed in the previous chapter and because of their chemical properties due to their composition, the organic substrates selected for hydrogen production in a Continuous Stirred Tank Reactor (CSTR) were the Jerusalem artichoke tubers and stalks. The Jerusalem artichoke tubers were mashed and stored at -20 °C for further feeding suspension preparation: each time, about 300 g of crude Jerusalem artichoke tubers were cut into small pieces and then mashed stepwise with 300 mL of distilled water in a blender for 2 minutes (Philips Compact Blender HR2027). Then, on the mashed suspension (< 2mm), moisture content, total solids as well as volatile solids contents were determined according to the methods described in paragraph 2.3.1.1. For preparing the tuber suspension ready for feeding the sCSTR, an appropriate quantity of meshed sample was thawed and diluted with distilled water to reach either 10 g total solids.L^{-1}, or 20 g total solids.L^{-1}. Then, one mL of concentrated oligo-element solution was added to reach the following final concentration (per liter): NH_4Cl 1000 mg, K_2HPO_4 150mg, $MgCl_2 \cdot 6H_2O$ 150 mg, NaCl 1000mg, $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ 25 mg, $ZnCl_2 \cdot 2H_2O$ 10 mg, $MnCl_2 \cdot 6H_2O$ 20 mg, $CuSO_4 \cdot 5H_2O$ 5 mg, $CoCl_2 \cdot 5H_2O$ 5 mg, $N_iCl_2 \cdot 6H_2O$ 20 mg, $CaCl_2 \cdot 2H_2O$ 10 mg (adapted from Aceves-Lara *et al*., 2007). The feeding solution was finally acidified to pH 5.5 with 37 % HCl before filling up the feeding tank.

II.2 Biological processes used for biohydrogen production

II.2.1 Biological Hydrogen Potential (BHP) test

The biological hydrogen potential (BHP) test is a batch experiment in which the substrate, *i.e*. organic solid waste, is fermented by a mixed culture under optimal conditions to produce a maximum amount of hydrogen, concomitantly with carbon dioxide and soluble metabolic products (Figure II-1). The biohydrogen potential batch experiments were carried

out at 37 °C in sealed 600 mL plasma flasks with no agitation. In order to keep the organic load an optimal range according to the results presented in chapter III-1.1.4., each flask contained from 1 to 9 gTS of the tested substrate. Then, 200 mL of MES buffer (2-[N-morpholino] ethane sulfonic acid, 40 mmol.L^{-1}) and 3 mL of seed sludge (final concentration of 225 mg-$_{COD}$.L^{-1}) were added to each flask. The seed inoculum corresponded to the sludge outlet of an anaerobic digester treating vinasses. The inoculum was heat treated at 90 °C for 10 minutes to deactivate methanogens prior to inoculation. After heat-shock treatment, the inoculum was stored at -20 °C to provide the same inoculum to all BHP experiments. All micronutrients were provided by the inoculum, and no additional nutrient medium solution was added. The initial pH value was adjusted to 5.5 with NaOH 2N or 37 % HCl. The headspace of the flasks was flushed with nitrogen for 5 minutes to provide anaerobic conditions. Once no more outlet biogas was produced and the hydrogen content started to drop off through the monitoring of the biogas composition, the experimental procedure was considered to be ended. The subsequent stage of hydrogen consumption then started, where the hydrogen produced was much less than the amount of hydrogen consumed. Each experiment was performed in duplicates.

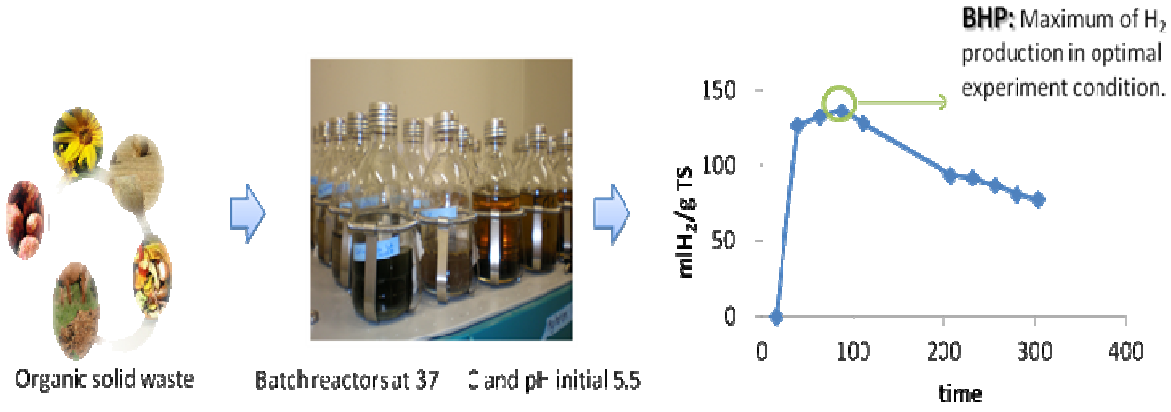


Figure II-1. Schematic representation of the biochemical hydrogen potential (BHP) tests.

II.2.2 Operation of Continuous Stirred Tank Reactor (CSTR) for bioHydrogen production

II.2.2.1 Instrumentation of sCSTR bioreactors

Two continuous stirred tank reactors (CSTR, Applikon®) of 3 L total volume were carried out in a continuous fermentation mode with a working volume of 1.5L. They were performed in duplicates. A schematic representation of the experiment is showed in Figure II-2. The reactor was equipped with a magnetic stirring contactor connected to an impeller shaft with a Rushton turbine (6 blades). The rotational speed of the stirring system was maintained at 350 rpm, periodically controlled with an external reference (digital-hand tachometer ROTARO™). Three baffles were placed near the walls of each reactor to maximize the mixing efficiency. These reactors were considered as completely mixed reactors. For each reactor, a pH meter (InPro® 3253i/SG/225, Mettler Toledo, Switzerland), a temperature sensor (Pt100), and a manometer (Keller mano2000, Switzerland) were connected to monitor continuously the experimental environmental parameters. For the acquisition of the data, the signals were sent to an input/output module TES (Leroy Automation, France) which converted the digital signal to an analog one and send the data to an acquisition software (MSPC™, version 2.1.c). The data were analyzed with a Matlab™ –based PID controller which activated the regulation pumps (Masterflex L/S, 18 tubing) according to the pressure (1 bar absolute) and pH (5.5) sets. Consequently, the pH was kept constant at 5.5 with a double regulation of NaOH (2N) and HCl (2N). The temperature was regulated with a silicone wrapped blanket around the reactor vessel to keep 37 °C, with an external regulator (MCI™ regulator – model AC1-5). The feeding solution was kept in an alimentation tank equipped with a mechanical stirring system at 4 °C to prevent pre-fermentation of the substrate. The feeding pump was periodically activated by the MSPC™ v.2.1c software once every 30 minutes during 20 seconds each time. This frequency of the feeding pump was defined to keep an HTR of 6 h according to the fixed flow rate of the peristaltic pump (Masterflex®, masterflex L/S, n°18 tubing). The working

volume of the reactor was kept constantly at 1.5 L by a level sensor: when the liquid increased in the reactor after inlet addition, the level sensors entered in electric contact and the signal switched an electronic relay to activate a high-speed withdrawal pump (Masterflex®, masterflex L/S, n°18 tubing) until the liquid level returned to the initial volume of 1.5L (Figure II-2). All online measurements were then recorded in a mySQL-based database, using a data and information management system (SILEX) developed in the LBE-INRA laboratory. This system allows viewing and retrieving data from processes.

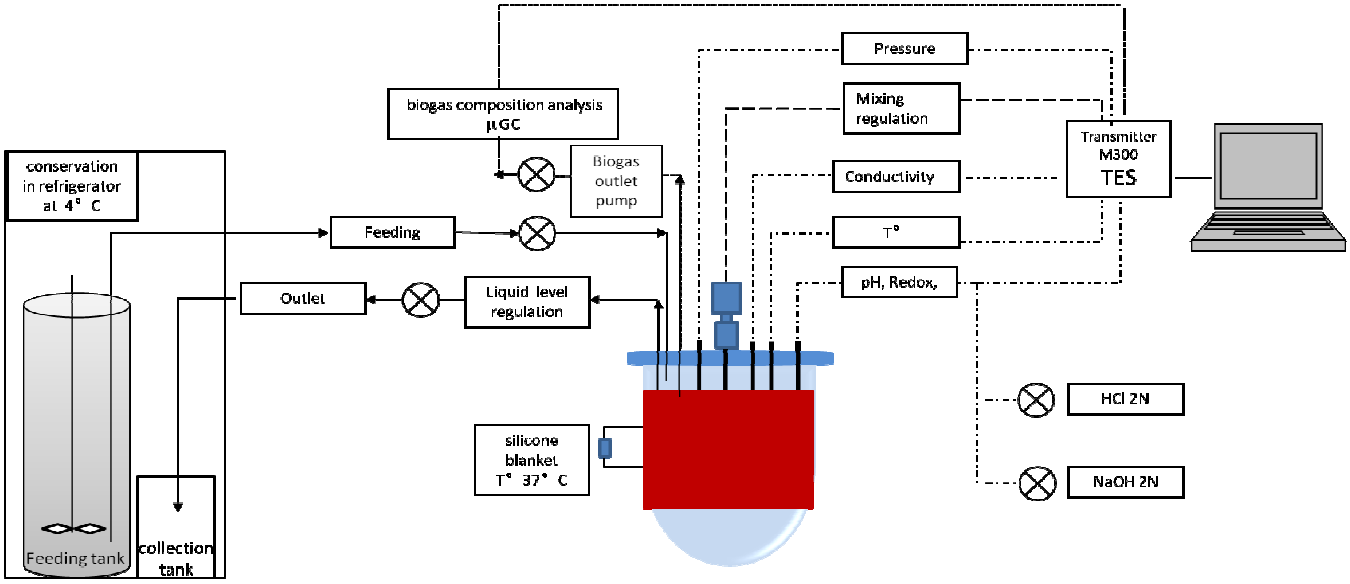


Figure II-2. Schematic representation of the continuous hydrogen reactor instrumentation.

II.2.2.2 **Start-up and operation in CSTR bioreactors**

Two series of experiments in CSTR operated in continuous mode were carried out in duplicates. In the first experiment, two concentrations of Jerusalem artichoke tuber solutions (*i.e.* 10 $g_{TS}.L^{-1}$ and 20 $g_{TS}.L^{-1}$) were tested. To start-up the fermentation system, the reactor was filled with 1.5 L of feeding solution of 10 $g_{TS}.L^{-1}$ and 1.5mL of mineral nutrition solution. No inoculum was added at the begining into the feeding solution to avoid the presence of hydrogen consuming microorganisms. The reactor headspace was flushed with nitrogen gas for 20 minutes to ensure anaerobic conditions at the start-up of the experiment. The pH and temperature were maintained at 5.5 and 37 °C, respectively. After 22 h of incubation in batch mode, the continuous operation of the reactor started with an HRT of 6 h. The operation parameters were kept constant as described in section II-2.2.1. The Jerusalem artichoke tuber feeding solution at 10 $g_{TS}.L^{-1}$ was added during the first five

days and shifted to a feeding solution at 20 $g_{TS}.L^{-1}$ the last five days. In this experiment, the mineral nutrition solution (see section II-1.2) was added in the feeding solution.

In the second experiment, the Jerusalem artichoke stalk solution and the mixture of tuber pulp and stalk were used as inlet feeding solutions. To start-up the fermentation system, the reactor was filled with 1.5 L of stalk solution at 13 $g_{TS}.L^{-1}$ with no mineral nutrition solution added. The feeding concentration of stalk was defined by a previous anthrone test results, with the purpose of having the same sugars equivalent to the 10 $g_{TS}.L^{-1}$ of tubers. The pH was adjusted to 5.5 and temperature was kept at 37 °C. The reactor headspace was flushed with nitrogen gas for 20 minutes. The batch fermentation mode was operated for 22 hours. And then the system was operated as a continuous fermentation system with an HRT of 6 h, a pH of 5.5 and a temperature of 37 °C. During the first 9 days, the feeding solution corresponded to Jerusalem artichoke stalk at 13 $g_{TS}.L^{-1}$, and during the next 9 days, the feeding solution corresponded to a mixture of Jerusalem artichoke stalks and tubers (6.5 $g_{TS}.L^{-1}$ of stalk and 5 $g_{TS}.L^{-1}$ of tubers). In this experiment, no mineral nutrition solution was added in the feeding. The exact HRT was periodically assessed through the measurement of the outlet volume sampled over time.

II.3 Analytical methods

II.3.1 Physico-chemical characterization of the organic substrate

II.3.1.1 Total solids (TS) and volatile solids (VS)

Porcelain crucibles were placed in an oven at 104 °C for at least one hour. They were then placed into desiccators until cooling at room temperature, and then weighed. Around 2 g of each sample were added in the porcelain crucibles, then weighed, and dried in an oven at 104 °C for 24 hours, until reaching a constant weight. After transferring them into a desiccator for cooling, these crucibles were re-weighed, and the difference corresponded to

the moisture content. The total solid content (TS) corresponded to the difference in percent of the weight after 24h drying and the initial sample weight (APHA-AWWA-WEF, 1995).

After TS measurement, the crucibles containing the dried samples were placed in an oven at 550 °C for 3 hours. The volatile solid (VS) content corresponding to the combusted organic matter was then determined after weighing the final ash content by difference with the TS content (APHA-AWWA-WEF, 1995).

II.3.1.2 **Determination of carbohydrate composition**

First, the determination of carbohydrate contents in solid waste using standard methods failed to hold a good repetition when measured in suspension, mainly due to the high heterogeneity and practical experimental constraints. We therefore decided to hydrolyze the part of complex carbohydrates in strong acids before quantifying the simple sugar units released. Firstly, a solution of H_2SO_4 at 72 % was tested. This strong sulfuric acid solution is commonly used to hydrolyze the cellulose fraction (see section II-3.1.4). Around 500 mg of samples were added to 40 mL of H_2SO_4 72 % and kept for 1 h in an ultrasonic tank (120W, 47 KHz) (Bransonic®, USA). The observation during the experiments showed that the colors of the acid solutions containing the substrates to be analyzed turned into a brown-black color, especially the Jerusalem artichoke plant. This was due to HMF (5-hydroxymethyl-furfural) generation during the hydrolysis procedure from fructose, the main monocarbohydrate found in Jerusalem artichoke (Nguyen, 2009). The changing color of samples influenced the further anthrone-reaction method based on colorimetric analysis. In order to avoid this undesirable effect obtained from concentrated sulfuric acid, other acids were tested with lower concentrations. Using insoluble starch as a reference, the use of 2 N hydrochloric acid with the same method (1 h in ultrasonic tank) showed satisfying results. All the liquid after this treatment presented only little change in colors. The sugar solubilization efficiency from starch was ranging between 99 ± 2 %.

Consequently, the method with 2 N hydrochloric acid was used for further experiments to solubilize the tested substrates. Practically, the overall carbohydrate-determination protocol was as follows: 500 mg of samples were added to 40 mL of 2 N HCL in a sealed glass vial and put one hour in an ultrasonic tank (120 W, 47 KHz) at room temperature. The liquid fraction was then diluted and centrifuged (20 000 g, 10 min). The supernatants were kept for further quantification assay with the anthrone method.

To determine the carbohydrate concentrations in the supernatants, the anthrone method was used according to the defined standard method (Dreywood, 1946). This method aims to assess the total reducing sugars content in liquid samples. It is based on the intramolecular dehydration of monosaccharides in acid medium at high temperature. Various forms of furfurals (*e.g.* 5-hydroxymethyl-furfural for hexose) condense with the anthrone reactant (9-oxo-dihydro-10-anthracene) to obtain colored products (*e.g.* green for hexoses) (Dreywood, 1946). A standard curve using glucose (from 0 to 200 $mg.L^{-1}$) was freshly prepared in order to assess precisely the sugar concentration in the samples. The concentration of carbohydrates was expressed in glucose equivalents (in $g_{eq.Glu}.L^{-1}$). The advantage of this method is that proteins, fats or acetate do not interact with the assays (Raunkjaer *et al*., 1994). In counterpart, the main disadvantage of this technique is the difference in sensitivities on various monosaccharides (Feller *et al*., 1991). In practice, 1 mL of liquid sample and 2 mL of anthrone reactive solution (2 % anthrone in H_2SO_4) were introduced in test tubes. The reaction started when the test tubes were incubated in a water bath at 100 °C for ten minutes. The reaction was stopped by immersing the tubes in ice. Then, 225 µL of the sample were transferred to a 96 well assay plate. The absorbance of the samples was then measured at 625 nm using a microplate spectrophotometer (Tecan - Infinite 200 NanoQuant).

II.3.1.3 **Determination of protein composition**

II.3.1.3.1 *Bicinchoninic Acid Method*

As the carbohydrate content in solid waste, the protein composition in solids cannot be directly determined. In this thesis, the protein content was firstly solubilised with a sodium hydroxide solution. For this, about 500 mg of substrate were treated with 40 mL NaOH 0.5M in an ultrasonic tank (120W, 47 KHz) for one hour. The supernatants were then centrifuged (20 000g, 10min). The protein concentrations were measured Bicinchoninic Acid Protein Assay Kit (BCA1) (SIGMA). The principle of this measurement is based on the formation of the complex "Cu^{2+}-protein" in alkaline conditions, followed by a reduction of Cu^{2+} to Cu^{+}. The quantity of reduction poor irons is proportional to the protein concentration in the sample. The BiCinchoninic acid (BCA) reacts with Cu^{+} forms, a blue color product which can be spectrophotometrically measured at 562 nm. The protein concentrations are determined from a standard curve made with bovine serum albumin (Sigma A7906). This analytical method from the BCA is more sensitive and more practical methods of Biuret and Lowry (Raunkjaer *et al*., 1993).

II.3.1.3.2 *Estimation of protein content from Total kjeldahl Nitrogen (TKN) determination*

According to Izhaki (1993), the protein content was estimated by multiplying the total organic nitrogen by a factor of 6.25 (Izhaki, 1993). Indeed, the total Kjeldahl nitrogen content is the sum of organic nitrogen, ammonia (NH_3), and ammonium ions (NH_4^+) concentrations in samples. In this method, the organic nitrogen was firstly transformed to ammonium sulfate by adding acid (H_2SO_4 + catalyst Kjeldah) in a mineralisator (BUCHI digestion unit K 438). The color change of the sample solution from dark to clear and colorless meant a total mineralization of the sample. This process could last for 4 to 5 hours. The second step was performed in a BUCHI 370-K distillator/titrator equipped with an auto-sampling system. The mineralized sample in form of $(NH_4)_2SO_4$ changed to NH_3 by adding sodium hydroxide (32 %). Then, the ammonia was trapped into a boric acid solution

(4 %), with a pH adjusted to 4.65 with NaOH (32 %). The ammonia reacted with the boric acid and the remained boric acid was then titrated with an acid solution of 0.02 N HCl. The quantity of reacting boric acid corresponded to the total organic nitrogen amount of the sample.

II.3.1.4 **Substrate fractionation according to the Van Soest Method**

The principle of this method is to fractionate the organic matter based on successive physico-chemical extraction steps (Van Soest, 1963). At each step, the samples were extracted with a detergent or an acid to remove the desired fractionated compounds (Figure II-3). The samples were dried and weighed after each extraction in order to quantify by weight each fraction i.e. soluble-like, cellulose-like, hemicelluloses-like and lignin-like contents. The assay was carried out in a FIBERBAG system (Gerhardt). The solid samples were first milled (<3mm) using a cutting miller (IKa Werke MF10). About 1 g of milled sample was then introduced in a FiberBag (Gerhardt) in a beaker, as support, previously dried and precisely weighted. The bags containing samples were in contact successively with three detergent solutions, and before and after each fraction, the weight of the bags with the beaker support were precisely measured: First, the bags were boiled for one hour in a neutral detergent solution (NDS) corresponding to 30 g of sodium dodecylhydrogenosulfate ($C_{12}H_{25}NaO_4S$)+ 6.81 g of sodium tetraborate ($Na_2B_4O_7$,$10H_2O$) +18.61 g of EDTA (Ethylenediaminetetraacetic acid) + 4.56 g of Na_2HPO_4, $5H_2O$ diluted in one liter of distilled water. At this stage, the “soluble-like” fraction was extracted. After having rinsed thoroughly with hot distilled water the undissolved sample, the bags were dried in an oven at 105 °C and weighed. Second, the “hemicellulose-like” fraction, soluble in acid detergent (ADS), was extracted by adding an acid detergent composed of 20 g of hexadecyltrimethylammonium bromide ($CH_3(CH)_{15}N(Br)(CH_3)_3$) + 28.8 ml of 96 % sulfuric acid (H_2SO_4), diluted in one liter of distilled water. Third, after drying and weighing, the bags were immersed in 40 mL of 72 % sulfuric acid for 3 hours to remove the “cellulose-like” fraction. Finally, the remaining fraction corresponding to the insoluble

fraction of any detergents was defined as the "lignin-like" fraction, determined afterwards in an oven of 550 °C for three hours and by weighing.

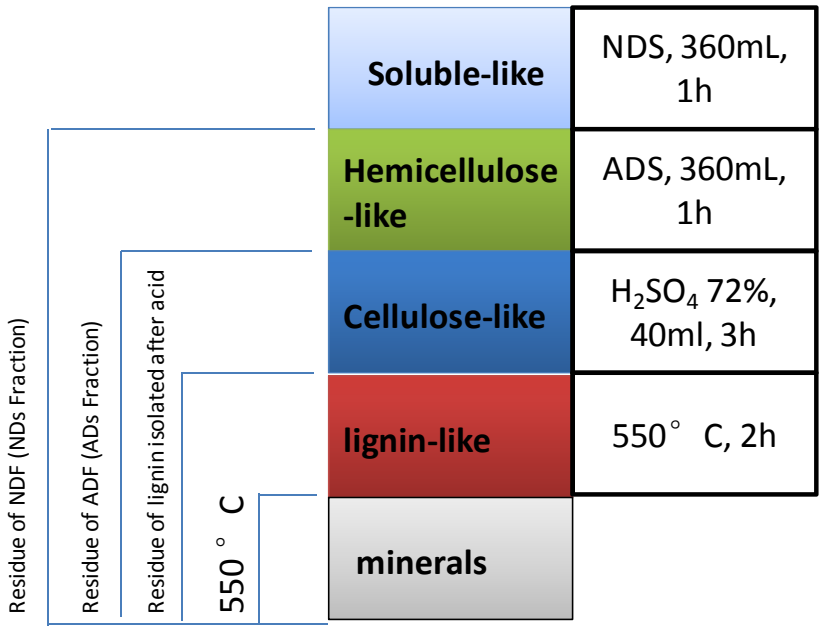


Figure II-3. Schematic representation of the Van Soest method for lignocellulosic substrate fractionation.

II.3.2 Bioprocess parameters analysis

II.3.2.1 Biogas volume measurement and composition analysis

II.3.2.1.1 *Biogas analysis in batch reactors*

Total volume of biogas produced in batch BHP tests was daily measured by using a water displacement method determined by the volume of acidified water displaced (pH=2). Acidified water at pH 2 (with HCl 37 %) was used to avoid the dissolution of CO_2 in water. Biogas measurement system is showed in Figure II-4. All the measurements of the porduced biogas volume had been performed at 25 °C and 1 atm. The factor to calculate to standard condition (0 °C, 1 atm) is 0.9161. After having determined the total volume of the biogas

produced, 1 mL of biogas was directly sampled from headspace to measure the biogas composition using a gas chromatograph (Shimadzu GC-8A) connected to a C-R8A integrator and equipped with a CTRI Alltech column. The following gases were measured: CO_2, H_2, O_2, N_2 and CH_4. The column was made up of 2 concentric columns. The 3.175 mm-diameter inner column was filled with Sillicagel. It allowed the separation of CO_2 from the other gases. The other gases were separated in the 6.350 mm-diameter outer column filled with a molecular sieve. The carrier gas was argon at 2.8 bars. The temperatures were 30 °C for the oven and 100 °C for the injector and the detector. The detection of gaseous compounds was done using a thermal conductivity detector and the intensity of current was 80 mA. The calibration was done with a standard gas composed of 25 % of CO_2, 5 % of H_2, 2 % of O_2, 10 % of N_2 and 58 % of CH_4.

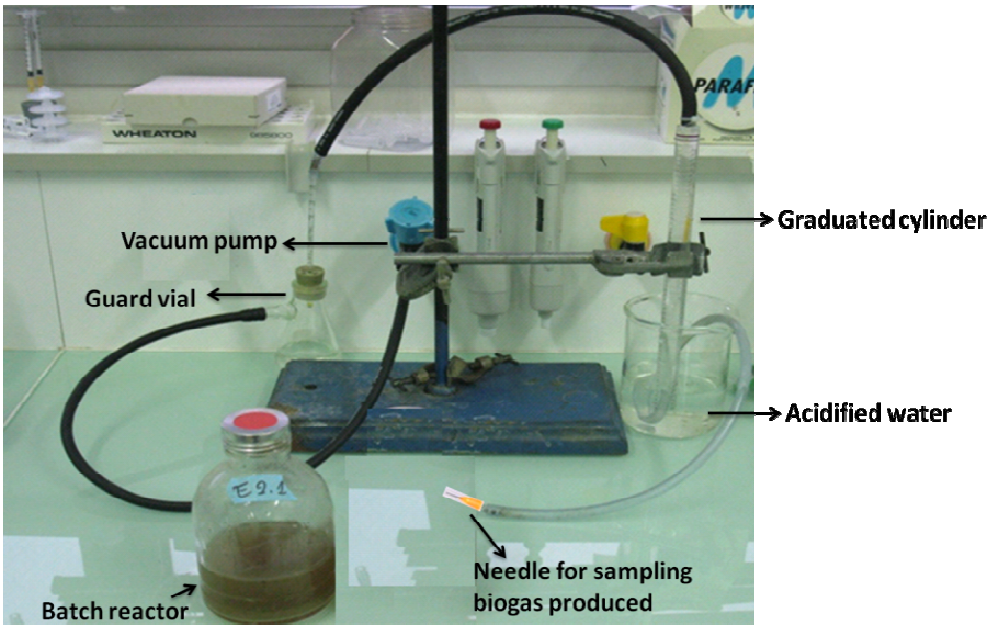


Figure II-4. Measurement system of the biogas produced in batch reactor.

The cumulative hydrogen gas production was calculated using a mass balance on each step which led to the following equation (II-1):

$$V_{H2,i}=V_{H2,i-1}+V_p.C_{H2,i}+V_{h,i}.C_{H2,i}-V_{h,i-1}.C_{H2,i-1} \quad \text{(II-1)}$$

where $V_{H2,I}$ corresponds to the cumulative hydrogen volumes (in mL) calculated at time "i" according to the cumulative hydrogen volume at time "i-1", *i.e.* $V_{H2,i-1}$; V_p the biogas volume measured by the water displacement method at time "i"(ml); $V_{h,i}$ and $V_{h,i-1}$ the total volumes of bottle headspace at days "i" and "i-1",respectively, *i.e.* considering the volume of liquid sampled over the experimental time; and $C_{H2,i}$ and $C_{H2,i-1}$ the hydrogen percentages in bottle headspace at days "i" and "i-1", respectively (in %).

II.3.2.1.2 *Assessment of biogas production in continuous reactors*

When biogas was produced in CSTR, the increased pressure values were measured by the manometer (Keller Mano2000, Swiss) connected to the reactors, and the data were acquired via TES to the on-line controlled computer. The pressure regulation was fixed at 1 bar and a peristaltic pump was activated to keep constant the pressure and evacuate the extra biogas produced in the reactor. According to the pump calibration, the total biogas volume and therefore productivities were calculated based on the rates of use of the pressure pump. Since the room temperature was constantly at 25 °C, the volume of produced biogas was therefore assessed at this temperature. The factor to calculate to standard condition (0 °C, 1 atm) is 0.9161.

A multiplexed micro-GC (R3000, SRA instruments, France) was directly connected to the gas outlet of the reactors for measuring online the biogas composition (Figure II-5). The Micro-GC R3000 was equipped with two columns: one micro-MolSieve 5Å column (10 m × 0.32 mm) with argon as carrier gas was used to detect CO_2 and the other Q PLOT column (8 m × 0.32 mm) with helium as carrier gas was used to detect O_2, H_2, N_2 and CH_4. The injector temperature was fixed at 90 °C. The temperature of the columns was maintained at 80 °C and the carrier gas pressure was fixed at 30 psi in both columns. The detector corresponded to a thermal conductivity detector (TCD). Each analysis was carried out over 180 seconds. The data were analyzed through the Soprane software (SRA Instruments®) to

assess the composition of the injected gas in mmol based on an external calibration. All data were collected in an internal mySQL database called SILEX, for any further analysis.

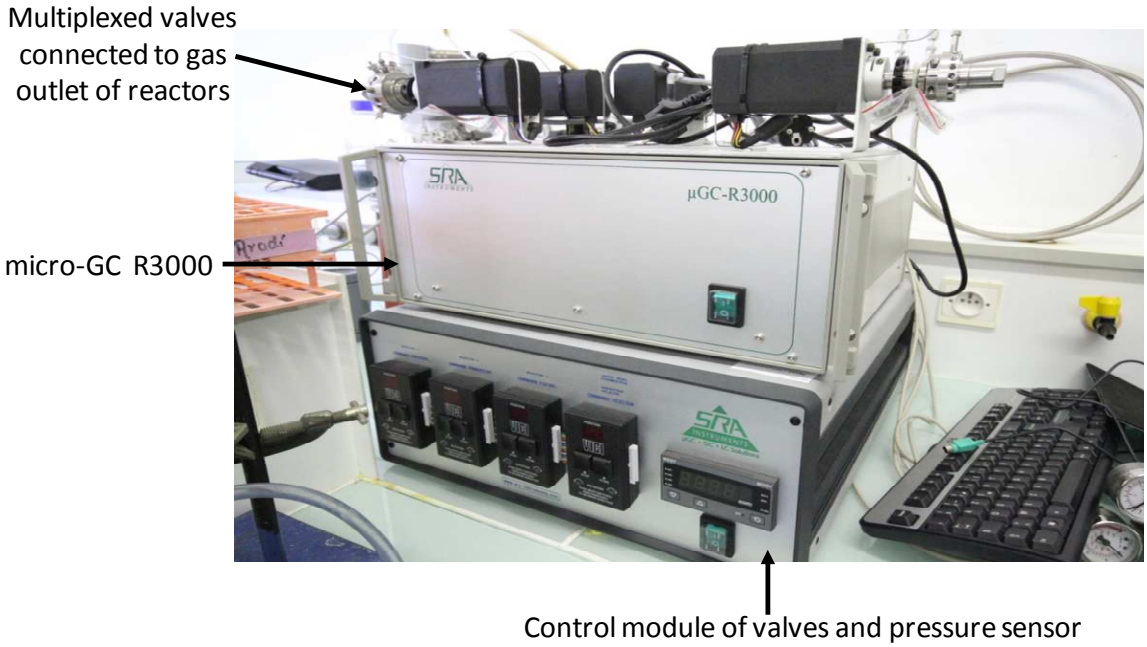


Figure II-5. Multiplexed micro-GC (R3000, SRA instruments, France) for measuring composition of biogas online.

II.3.2.2 **Metabolic end-product analysis**

II.3.2.2.1 *Volatile fatty acids (VFAs) analysis*

The liquid samples were collected in 2 mL Eppendorf® tubes and were then centrifuged at 11 337g for 15min. Afterwards, 500 µL of the supernatant were transferred in analytical vials where 500 µL of standard internal solution (1 $g.L^{-1}$ of diethylacetic acid ($C_6H_{12}O_2$) acidified to 5 % with H_3PO_4) was added. These samples were then analyzed by gas chromatography coupled to flame ionization detection (GC-FID) in a Varian GC 3900 chromatograph equipped with an auto-sampling system (Middelburg, The Netherlands)

Figure II-6). Basically, the VFAs compounds of the samples are volatilized in the injector and then separated by affinity to the stationary phase materials within the column. The elution was carried out in a semi-capillary column FFAP of 15 m and 0.52 mm in diameter (Phase ECTM 1000). The carrier gas was nitrogen (Linde^{TM}, Nitrogen gas 5.0). The detection was performed with a FID (flame ionization detector) with H_2 (Linde^{TM}, hydrogen gas 5.0) as burning gas. The conditions of elution were as follows:

• Carrier gas: nitrogen, P = 20 psi

• Injector temperature: 210 °C

• Detector temperature: 280 °C

• Gas flow rate: 6 mL min^{-1}

• Range of oven temperature: 80 to 120 °C with a ramp rate of 10 °C per minute after 1 min of elution

Data acquisition was performed with the software Varian Galaxy Work Station (version 1.9.3.2).

Figure II-6. Varian gas chromatography 3900 and auto-sampling system for VFAs analysis (Middelburg, The Netherlands).

II.3.2.2.2 *High Pressure Liquid Chromatography (HPLC) analysis of the metabolic end-products*

Expect of VFAs, other fermentative products such as organic acids (*e.g.* lactate and formate), alcohols (*e.g.* ethanol) were quantified by high pressure liquid chromatography. After centrifugation of the reactor samples in Eppendorf® of 2 mL, 800 µl of supernatant were transferred to a vial prior to the analysis by high-pressure liquid chromatography (HPLC). A 0.2-µm filtration step (Nylon membrane, Acrodlsc ®) was performed when supernatant was not clear since the HPLC column was highly sensitive to micro particles. The analytical chain was composed of an automatic sampler (Water 717plus), a pumping system (DIONEX UltiMate 3000), an oven (DIONEX ultimate 3000RS) equipped with a protective precolumn (Microguard cation H refill cartbridges, Bio-Rad), a separation Aminex column (HPX-87H, 300x7.8mm), and a refractometer as detector (Waters996) (Figure II-7). The pre-column aimed to filter out the residual particles before flowing into the separation column. The compounds were thus separated using an Aminex HPX-87H column, 300 x 7.8 mm (Bio-Rad). The column was placed in an oven maintained at 35 °C. The isocratic elution consisted of H_2SO_4 6 mmol.L^{-1}, pumped at a rate of 0.4 mL min^{-1}. The refractometric detector temperature was fixed at 45 °C. The elution retention time of the different compounds are presented in Table II-1.

Figure II-7. High Pressure Liquid Chromatography (HPLC) system.

Table II-1. The different compounds analyzed by HPLC and their elution retention times (t_R).

Compounds		t_R (mins)
Name	Formula	
Glucose	$C_6H_{12}O_6$	13.89
Fructose	$C_6H_{12}O_6$	15.42
Lactate	$CH_3CHOHCOOH$	20.27
Formate	CHOOH	21.64
Acetate	CH_3COOH	23.16
Propionate	CH_3CH_2OOH	27.24
Ethanol	CH_3CH_2OH	31.25
Butyrate	$CH_3(CH_2)_2OOH$	33.45
Valerate	$CH_3(CH_2)_3OOH$	47.12
Caproate	$CH_3(CH_2)_4OOH$	76.5

II.4 Characterization of the microbial communities

II.4.1 Reactor sampling procedure

Duplicate liquid samples of 2 mL were collected from continuous reactor outlets twice a day and were centrifuged at 11 337 g for 20 min in 2 mL Eppendorf® tubes. The supernatant and the pellet were conserved separately at -20 °C for further metabolite (see section II-3.2.2) and microbial community analysis, respectively.

II.4.2 Molecular fingerprinting of microbial community

An overview of the different microbial ecosystems involved in H_2 metabolic networking was obtained by molecular analysis which consisted of (i) DNA extraction and

purification from the environmental samples,(ii) a PCR (Polymerase Chain Reaction) amplification of the V_3 region of 16S rDNA genes followed by (iii) a DNA PCR product separation by CE-SSCP (Capillary Electrophoresis-Single Strand Conformation Polymorphism). This technique provides the structure of the microbial community that could be further used to evaluate the diversity and the relative abundance of the present microbial population. Furthermore, CE-SSCP analysis provides accurate assessment of the microbial dynamics over experimental time.

II.4.2.1 **DNA extraction and purification**

The total genomic DNA was extracted from the sample pellets (see section II-4.1) using the Wizard Genomic DNA Purification kit (Promega). The procedure followed the instructions of the "isolation of genomic DNA of bacteria" protocol. The first step was performed to disrupt the cell walls. For this, the Eppendorf® tubes where the cell pellets were conserved, were incubated at 37 °C for 30 min after addition of 480 µL of EDTA (50 mM) and 120µL of lysozyme (10 $mg.mL^{-1}$). The tubes were then centrifuged 2 min at 10 000 g. After gently removing the supernatants, 600 µL of cell lysis solution was added, and the tubes were incubated at 80 °C for 5 min. Three µL of RNase solution were added afterwards, and the tubes were incubated again at 37 °C for 30 min. At this step, the membranes of the cells were totally solubilized. The proteins and other cellular impurities were removed using 200µL of precipitation solution (supplied by the extraction kit). After a vigorous mixing of 20 seconds, the tubes were placed in ice for 5 minutes and then centrifuged 3 minutes at 10000g. The supernatants containing the DNA were then collected in sterile 1.5 mL tubes.

The last step concerned specifically DNA purification. For this, 600 µL of isopropanol was added to each tube. After 2 minutes of centrifugation at 13000 rpm, the supernatants were carefully removed and the tubes were dried at room temperature for at least 10 min in order to evaporate all the isopropanol. The purification step was repeated with 600 µL of

ethanol at 70 %. After drying the DNA pellet for 10-15 min, 100 µL of DNA rehydration solution, provided by the kit, was added into each tube to solubilize the purified DNA. This step lasted for 1 hour at 65 °C or one night at 4 °C.

After DNA extraction and purification, the purity and quantity of DNA were assessed by absorbance measurement (OD-optical density) at 260 nm and 280 nm using a spectrometer (Infinite NanoQuant M200, Tecan). The ratio OD_{260nm}/OD_{280nm} was used as an indicator of the DNA purity, and should be ranging between 1.8 and 2. If the value was appreciably lower, it indicated a possible presence of compounds possessing a strong absorbance at 280nm such as proteins, phenols or other contaminants, all potential inhibitors of PCR amplification.

II.4.2.2 **DNA amplification by Polymerase Chain Reaction**

The V3 region of the gene encoding 16S rRNA (about 200 bp) was amplified by using Pfu Turbo DNA polymerase (Stratagene), with a target to all bacteria using the bacterial primer W49 (5′-ACGGTCCAGACTCCTACGGG-3′, *Escherichia coli* position F331) and the universal primer 5-fluorescein phosphoramidite-labeled W104 (5′-TTACCGCGGCTGCTGGCAC-3′, *E. coli* position R533) (Delbes *et al.*2001). The primer W104 was labeled with a fluorochrome (6-FAM^{TM}: 6-carboxyfluorescein) for further detection of the amplified fragment by fluorescence detection in the CE-SSCP equipment.

PCR amplification was performed in a Mastercycler thermal cycler (Eppendorf®), with 50 µL of reaction mixture including 36.9 µl of pure H_2O, 5 µl of Pfu Turbo 10x buffer, 4µl of dNTP (2.5 mM), 1.3 µl of W49 (100 ng.μL^{-1}), 1.3µl of W104 (100 ng.μL^{-1}), 0.5µl Turbo Pfu (2.5 U.μL^{-1}) and 1 µl of DNA. The amplified cycles were as follows: initial denaturation for 2 min at 61 ℃, followed by 25 cycles of 94 ℃ for 30s, 57 ℃ for 30s, and 72 ℃ for 30s, and a final extension at 72 ℃ for 10min.

The PCR amplification was verified by using the Bioanalyzer 2100 (Agilent), which allows the analysis of PCR products by miniaturized electrophoresis based on separating DNA strands and according to their molecular weights. Basically, in this technique, the separation takes place in a mini gel matrix (products provided in kit), and negatively charged nucleic acids are electrophoretically driven under an electrical field (30W). The smaller the DNA molecule is, the faster and the longer distance they would migrate. A dimethyl sulfoxide (DMSO) marker added in the DNA fragments allowed the detection by a UV laser. At this stage, the intensity of the bands was used as an indicator of the efficiency of the PCR. The amplified DNA samples were conserved at -20 °C prior to CE-SSCP analysis.

II.4.2.3 **CE-SSCP (Capillary Electrophoresis-single Strand Conformation Polymorphism) analysis**

II.4.2.3.1 Principle*:*

The CE-SSCP (Capillary Electrophoresis-single Strand Conformation Polymorphism) analysis corresponds to a molecular fingerprinting technique which provides an instantaneous picture of the structure and the diversity of the microbial ecosystem. In this technique, the DNA fragments, previously amplified by PCR with fluorochrome labeled primers, are first denatured to their single-strand form with a thermal-shock. Each single-strand DNA fragment is then re-associated in ice to form a secondary structure according to the DNA sequence. This secondary structure leads to different separation migration speed in an electrophoretic capillary of an automatic sequencer (Figure II-8). The separated labeled strand DNAs are then detected by fluorescence after excitation by a laser and the fluorescence signal is recorded and displayed in a form of a peak in a chromatogram. Theoretically, one fluorescence peak corresponds to one unique DNA sequence, and by extension to one microbial species. The peak area is proportional to the amount of DNA detected, and by extension each peak area are reflecting the relative abundance of one unique species within the ecosystem (Delbes *et al*., 2001).

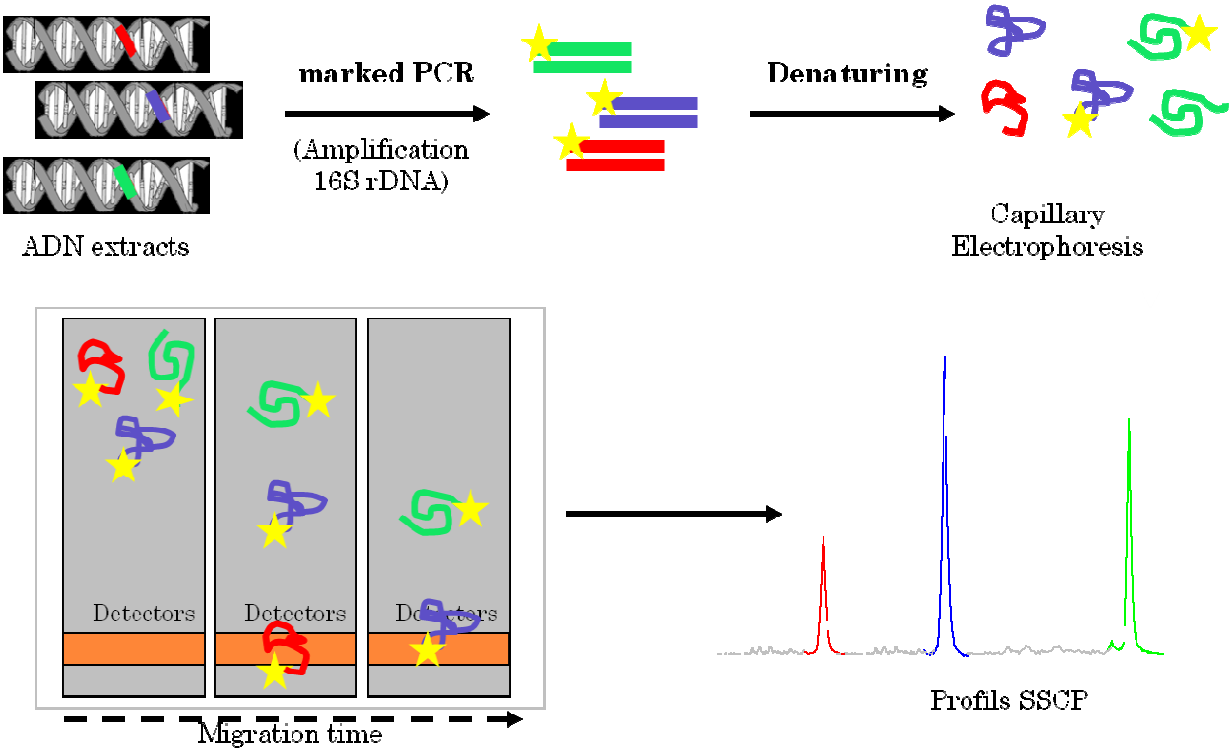


Figure II-8. Schematic representation of the CE-SSCP principle (from Dumont, 2008).

II.4.2.3.2 Technical Details:

Previously diluted from 5- to 2000-fold according to the intensity of the peak detected by the Bioanalyzer (Agilent Technologies, USA), one µl of the PCR amplified product was mixed with 18.8 µl of formamide and 0.2 µL of internal standard GeneScan ROX 400 HD (Applied Biosystems). The microplate containing the DNA samples was heated at 95 °C to denature the double strands DNA for 5 min and then immediately cooled on ice for 10 min. The CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems). The 16S rDNA fragments migrated by capillary electrophoresis in a capillary column (47cm × 50 microns) filled with a polymer (polymer GeneScan, Applied Biosystems) consisting of 5.6 %, 10 % glycerol, and 1 × TBE. The DNA fragment migration conditions were 12 kV and 32 °C for 30 min (Hitachi Applied Biosystem 3130 Genetic Analyser) (Figure II-9). Raw CE-SSCP data were analyzed using GeneScan

software (Applied Biosystems). The CE-SSCP profiles (*e.g.* Figure II-10) were then aligned with an internal standard ROX 400 HD to correct the slight differences in the electrophoretic migration between each run. The sum of the peak areas were normalized to unit before statistical analysis by the StatFingerprints library in R (R Development Core Team 2011) (Michelland *et al.*, 2009).

Figure II-9. Automatic sequencer 3130 (Hitachi Applied Biosystem, Japan).

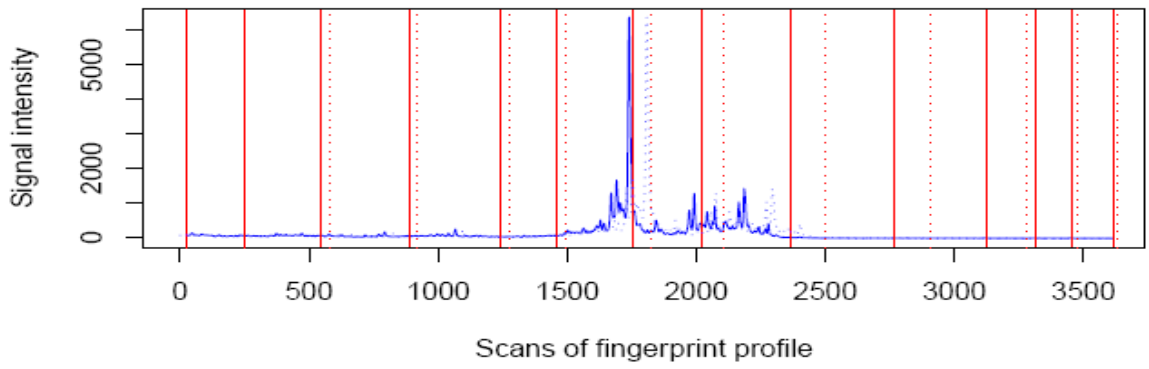


Figure II-10. An example of profile CE-SSCP. The red lines are the ROX marker migration peaks. The x-axisand y-axis correspond to arbitrary units.

In addition, several CE-SSCP data analysis were performed to characterize the microbial communities:

- The Euclidean Distances (ED) between two CE-SSCP profiles was calculated and used as quantitative indicators of dissimilarity between bacterial communities (Magurran, 2004). For this objective, le package “Vegan” in R was used.
- A statistic test ANOSIM was also carried out in “StatFingerprints”. The ANOSIM test provides a similarity analysis by comparison of the ED between different CE-SSCP profiles. A value of the coefficient R-ANOSIM was given and ranged between 0 (no difference) to 1 (fully different). This result was associated with a probability “p-value”, which is the probability of incorrectly rejecting the null hypothesis. The “p-value” indicated the significance of the statistic result. The lower “p-value” was, the more significant the statistic result was (Clarke, 1993).
- A principal component analysis (PCA) was also processed in R (Michelland *et al.*, 2009) by reducing and condensing the qualitative data. The PCA results were projected on a plan with clusters of comparative data. This grouping of profiles indicates a statistical similarity between them. Furthermore, such projection was ordered according to several environmental factors via vectors, to point out their influence to the microbial

community structure. The length of the vectors represented proportionally the importance of the influence.

II.5 Process modeling

II.5.1 Hydrogen performance modeling of the BHP test

Using a biogas mass balance for the flask headspace, the cumulative hydrogen production was determined for all batch experiments. A modified Gompertz Equation model (Eq.II-2) was fitted to the experimental data of cumulative hydrogen production as shown in Figure II-11.

$$H(t)=H_{max}\bullet.\exp\{-\exp[R\bullet e/ H_{max}(T_{lat}-t)+1]\} \qquad \text{(Eq.II-2)}$$

where Hmax corresponds to the maximum cumulated hydrogen amount (hydrogen potential) expressed in mL of hydrogen per gram of total solids, R the maximum hydrogen productivity (in $L.(L.day)^{-1}$), T_{lat} the lag phase (in days) and t the incubation time (in days). This model was fitted to the experimental data in Matlab (V6.5, Mathworks) using a non-linear regression algorithm.

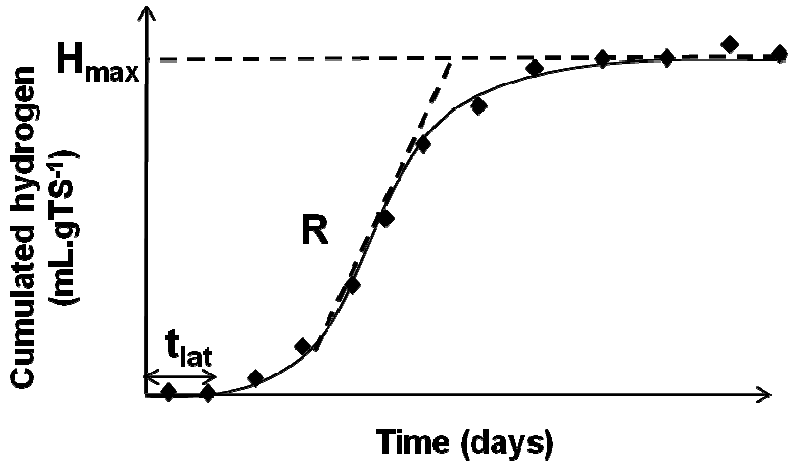


Figure II-11. Correspondence of fitted parameters of the modified Gompertz model (solid line) according to experimental data (solid points). The estimated values provided by the model are Hmax, R and t_{lat} which correspond to the hydrogen accumulated production ($ml.gTS^{-1}$), maximum hydrogen production rate ($ml.(L.day)^{-1}$) and the lag-phase time (days), respectively.

II.5.2 Multivariate analysis of experimental data and prediction of biological hydrogen potential with PLS regression

Partial least square (PLS) regression technique is based on constructing PLS factors (also called principal components) by minimizing the covariance between the dependent variables (Y block: BHP values) and the explicative variables (X block: substrate and product concentrations). Then, the prediction of the Y block was calculated with a multivariable linear regression on X block through PLS1 models using the software R version R 1.2.2 for Windows and by using PLS functions developed by Durand (1998). The algorithm constructs orthogonal PLS factors in each block by minimizing the covariance between the X and Y blocks. The first PLS factor contains the highest percentage of variance, and the following factors account for decreasing amounts of variance. A PRESS criterion (predicted residual error sum of square) was used in a leave-one-out cross-validation procedure to choose the dimension of the predictive model.

CHAPTER III. RESULTS AND DISCUSSION

Part of this chapter was adapted from GUO XM, LATRILLE E, TRABLY E, CARRERE H, STEYER JP. Substrate mapping by PLS regression analysis and prediction of biological hydrogen potential tests from solid organic waste. Waste management (submitted).

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III.1 Impact of organic solid waste composition and structure on biohydrogen production

Hydrogen production from organic waste by dark fermentation has gained significant interest over the past ten years, regarding the bio-energy research topics. A rapid increase of publications per year on hydrogen production by dark fermentation from organic waste is noticed in the ISI Web of knowledge, from 9 in 2005, 46 in 2008 up to 63 in 2011. However, the reported data, especially about the determination of hydrogen yields in batch tests, is highly dependent not only on the exact nature of one category of substrate but also on the experimental conditions (*e.g.* pH, temperature). As an example, food waste has been largely investigated as feedstock for hydrogen production and represents a wide variety of hydrogen yields likely due to variable composition and definition of such feedstock as well as the conditions of the tests (Guo *et al.*, 2010). It is thus difficult to analyse and compare these data in order to establish meaningful conclusions. The objectives developed of this chapter were to (i) develop a standard method to assess the hydrogen yield from organic substrates in batch tests, so called biochemical hydrogen potential (BHP), and (ii) to evaluate the impact of substrate composition and structure on the hydrogen yields and metabolic pathways of the mixed culture.

For this, a simple, robust and standardized method to evaluate the biochemical hydrogen potential from diverse organic substrates was optimized according to the operating conditions such as pH, buffer concentration and organic loading (this chapter, section III-1.1). Then, a large range of organic solid substrates, categorized in four types, was tested using the previously optimized protocol (this chapter, section 1.2). Meanwhile, a general method of characterization of substrate composition by hydrolysis and quantification of total carbohydrate and protein contents was applied to all types of organic solid substrates, and allowed to compare the relationships existing between such main substrate composition, hydrogen yields and metabolic pathways. An additional and more in depth analysis using the fractionation method of Van Soest was carried out to characterize the lignocellulosic substrates and to investigate which part of the carbohydrates within these substrates were bio-available to hydrogen production (this chapter, section III-1.2).

III.1.1 Optimization of the biochemical hydrogen potential (BHP) test

In this section, a protocol of biochemical hydrogen potential (BHP) batch test was optimized. This protocol will be further used as a standard method to evaluate the potential abilities of microbial communities on diverse organic substrates towards biohydrogen production. Thus, the protocol should meet several criteria such as quickness, easy operation and low-cost. A pre-test was previously developed in our laboratory (LBE-INRA, France), and several parameters were already defined, such as initial pH, microbial inoculum treatment, and the substrate/inoculum ratio. In this section, the impact of other operating parameters, such as the buffer selection and concentration, the oligo-nutrients addition and the substrate organic load were evaluated and optimized.

III.1.1.1 Impact of the type of buffer

pH is one of key parameters in bioprocesses and particularly in dark fermentation. Acid conditions have been widely reported to favour effective hydrogen production due to the favoured growth of efficient hydrogen-producing *Clostridium* sp bacteria, the effect on metabolic pathways and the unfavoured growth of hydrogen-consuming methanogens (Li and Fang, 2007). A range of 5.2-5.6 was considered as optimal pH range (Liu *et al.*, 2006), with, as illustrations, a pH of 5.2 for noodle manufacturing wastewater (Noike *et al.*, 2002), a pH of 5.5 for rice winery wastewater (Yu *et al.*, 2002), and pH 5.6 for food waste and sewage sludge (Lay *et al.*, 1999). Since simple batch tests are operated without pH control, an appropriate buffer should be selected to keep acid fermentation environment close to an optimal pH. For this, a phosphate ($Na_2HPO_4.12H_2O$: 9mM; KH_2PO_4: 61 mM) and MES (2-(N-Morpholino) EthaneSulfonic acid; 20mM) buffers were compared in terms of hydrogen production performances. The neutral phosphate buffer is the most commonly used buffer in biochemistry with a pH range of 5.8-8.0, while the MES ($C_6H_{13}NO_4S$), widely used in enzymatic tests, was here tested for its more acidic pH range of 5.5-6.7 (Good *et al.*, 1966).

Duplicates on each buffer were carried out at 37 ºC in dark fermentation with heat-schock treated anaerobic inoculum, and with glucose at 10 $g.L^{-1}$. Figure III-1

illustrates the cumulative hydrogen production observed with the two buffer solutions. The total volumes of cumulative hydrogen production were 118 ± 36 $ml_{H2}.g_{glucose\ added}^{-1}$ and 231 ± 8 $ml_{H2}.g_{glucose\ added}^{-1}$ for phosphate and MES buffers, respectively. The BHP test in presence of MES buffer led to almost a double hydrogen volume, which was probably linked to pH evolution over the experimental time. Indeed, although the initial pH was ranging 5.5 to 6 (with no effect in this range on final hydrogen yield – data not shown), the final pH were 3.53 with MES buffer, and 4.42 with phosphate buffer. At this pH, hydrogen production was probably stopped with no further evolution as shown in Figure III-1, which is consistent with a previous report made on starch where no hydrogen production was observed at pH 4.0 (Liu and Shen, 2004). With a pKa of 7.2, the phosphate buffer was probably not efficient to keep the solution at a good range of pH (5.5-6) for optimal biohydrogen production. In contrast, with a pKa of 6.1, the MES buffer provided favourable conditions for biohydrogen production pathways. This assumption is consistent with the observations of the different metabolic pathways, as shown in Figure III-2. While the final soluble metabolic products with MES buffer were only acetate and butyrate which correspond both to hydrogen-producing pathways, another metabolic product, propionate, was generated in phosphate buffer, which corresponds to an unrecommended dissipative hydrogen pathway.

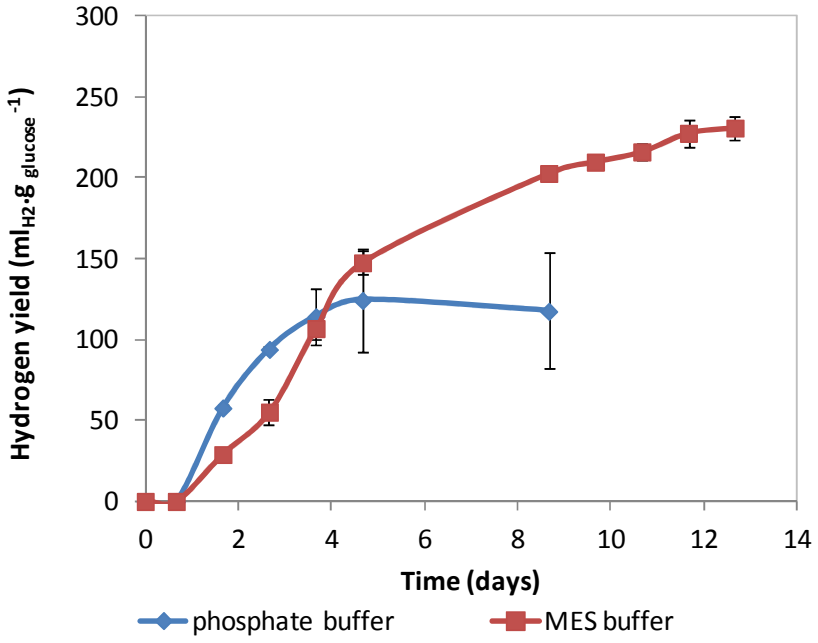


Figure III-1. Hydrogen yield from 10 $g.L^{-1}$ glucose in batch tests with phosphate ($Na_2HPO_4.12H_2O$: 9mM; KH_2PO_4: 61 mM) and MES (20 mM) buffers.

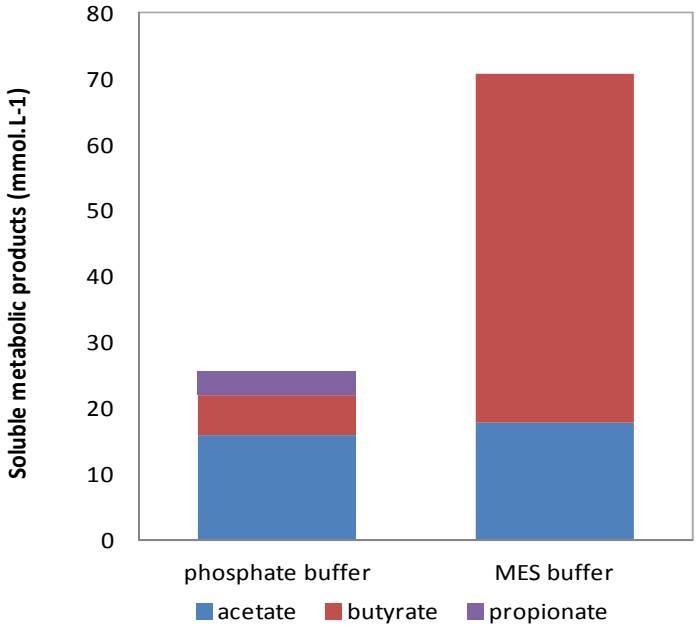


Figure III-2.Metabolic products from 10 $g.L^{-1}$ glucose at the maximum hydrogen accumulation time with phosphate ($Na_2HPO_4.12H_2O$: 9mM; KH_2PO_4: 61 mM) and MES (20 mM) in batch tests.

III.1.1.2 **Effect of MES buffer concentration**

A range of 20 - 80mM of MES buffer was tested on glucose at 10 $g.L^{-1}$. Duplicate cumulative hydrogen curves along the process at different concentrations of MES are presented in Figure III-3. As a result, it was observed that the maximum hydrogen yield increased when the concentrations increased from 20mM to 40mM with 184 ±7.7 $mL_{H2}.g_{glucose\ added}^{-1}$ and 239 ±9.3 $mL_{H2}.g_{glucose\ added}^{-1}$, respectively. At higher MES concentrations, *i.e.* 60mM and 80mM, the hydrogen yields were slight lower with 213 ±3.2 $mL_{H2}.g_{glucose\ added}^{-1}$ and 218 ±7.3 $mL_{H2}.g_{glucose\ added}^{-1}$, respectively. As expected, higher MES concentration led to higher final pH values, with pH around 3.5 at 20 and 40mM, and pH around 4.0 at 60mM and 80mM (Figure III-4). Meanwhile, higher hydrogen consumption was observed at higher MES concentrations at the end of the experiment. This suggests that H_2-consuming bacteria were more favoured at such higher MES concentrations due to a higher maintenance of the pH values. Only acetate and butyrate were detected, but also a continuously increase of the acetate

concentrations was observed for MES concentrations at 60mM and 80mM after the 9th day, implying the direct consumption of hydrogen to acetate by homoacetogens (data not shown). This is consistent with literature data where homoacetogenesis was reported at similar pH in batch tests with no regulation (Luo *et al.*, 2010).

In conclusion, the concentration of MES buffer was fixed at 40mM for further experiments.

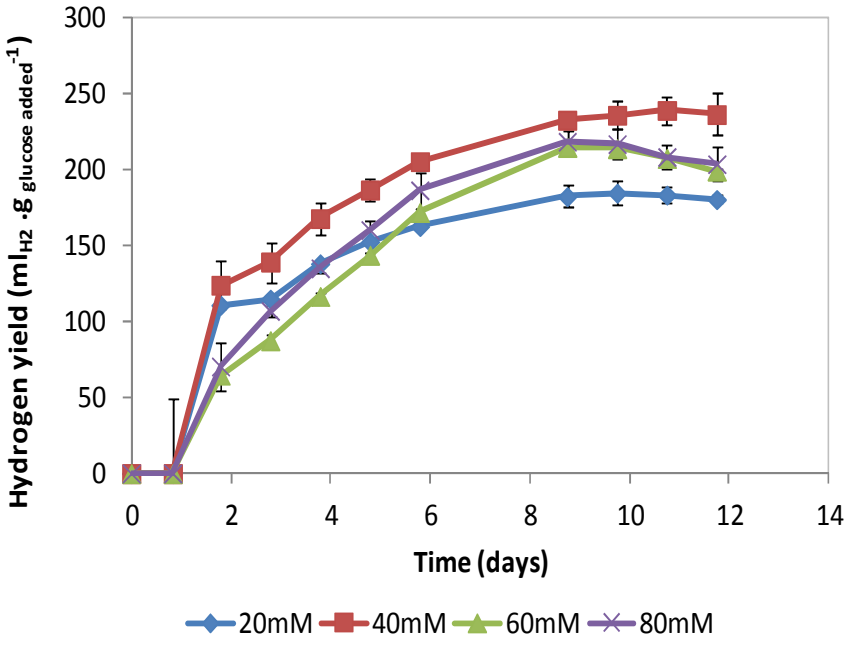


Figure III-3. Hydrogen yield from glucose at 10 g.L^{-1} according to the MES buffer concentration in batch tests.

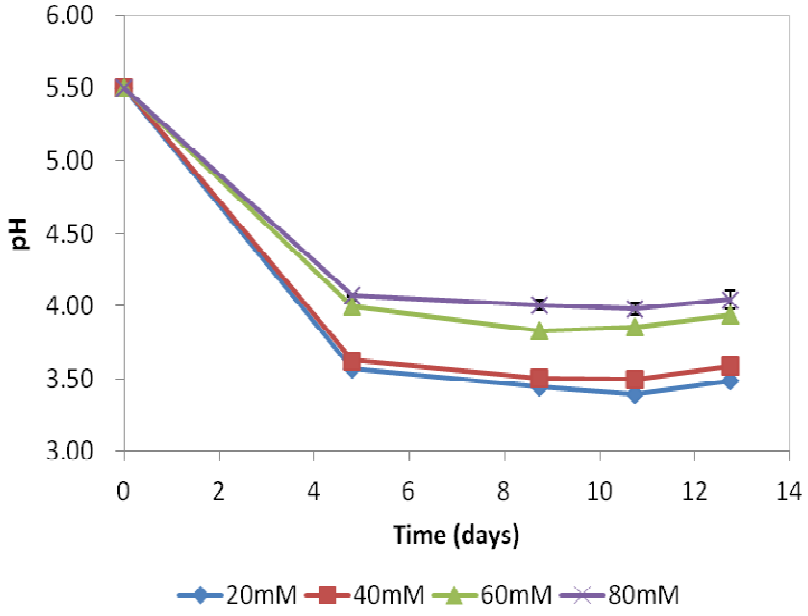


Figure III-4. pH evolution along fermentative hydrogen production in batch tests from glucose at 10 $g.L^{-1}$ according to the MES buffer concentration.

III.1.1.3 Effect of oligo-nutrients

In previous studies reported in the literature, an oligo-elements solution is usually added (Venkata Mohan *et al.*, 2012), considering the importance of nitrogen, phosphate and trace elements on bacterial growth and activity (Li and Fang, 2007). Therefore, in order to determine the effect of oligo-nutrients in batch tests, a comparison assay was carried out on glucose at 10 $g.L^{-1}$ with and without addition of an oligo-element solution. The oligo-element solution was adapted from Aceves-Lara *et al.* (2007) and is described in the "Material and Methods" chapter, section II-1.2. Figure III-5 presents the hydrogen yields from glucose at 10 $g.L^{-1}$ with and without oligo-elements. It was shown that no significant difference on hydrogen yield (*i.e.* 237 ± 5 $mL_{H2}.g_{glucose}^{-1}$ and 221 ± 15 $mL_{H2}.g_{glucose}^{-1}$ with and without oligo-elements, respectively), although maximum of cumulative hydrogen was reached faster in the trials with oligo-elements. Indeed, oligo-elements were related with the kinetic of hydrogen accumulation but did not significantly influence the final amount of hydrogen volume, the so-called hydrogen yield. Since the overall objective was to compare the hydrogen yields from different organic feedstocks including the eventual

lack of oligoelements, no oligo-elements were finally added although biohydrogen production rate could be impacted.

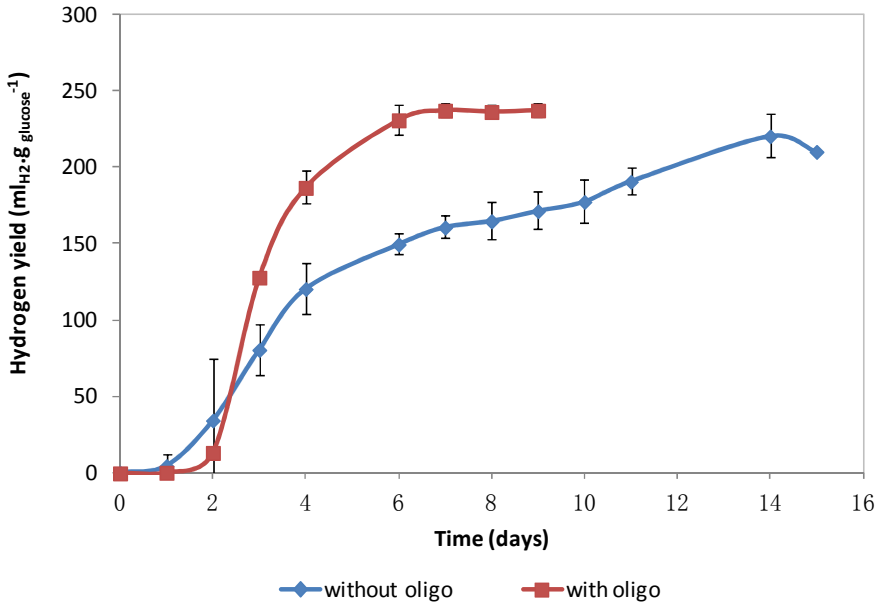


Figure III-5. Effect of oligo-elements on cumulative hydrogen production in batch test with glucose at 10 $g.L^{-1}$. The batch tests were carried out in duplicates at an initial pH of 5.5, a MES concentration of 40mM, and a temperature of 37 ºC.

III.1.1.4 **Effect of organic load**

Glucose as a model substrate was used to investigate the effect of organic load on hydrogen fermentation in batch test. In this experiment, the initial organic load was ranging from 1 $g.L^{-1}$ to 20 $g.L^{-1}$, with an initial biomass concentration of 225 $mg_{COD}.L^{-1}$. MES buffer of 40 mM was used and no oligo-nutrients were added. Each batch test was carried out in duplicates.

The curve of hydrogen accumulation over the experimental time is shown in Figure III-6. During the first 24 h incubation, a tiny volume of hydrogen was detected in only few several serum bottles. This step corresponded to the time of adaptation and growth of the initial microbial consortium, equivalent to a lag phase. A sharp increase in hydrogen accumulation was observed between 24h and 72h of incubation

for the initial glucose concentrations ranging from 2 $g.L^{-1}$ to 20 $g.L^{-1}$. The cumulative hydrogen in all the bottles decreased as soon as the curve reached the maximum accumulation by observing of no biogas production and decreasing hydrogen content in the headspace (data not shown). This indicated the consumption of hydrogen by the bacteria from the mixed culture. The accumulation of hydrogen stopped at 2, 3, 4, 8, 15, 14 and 15 days for the 1, 2, 4, 8, 10, 16 and 20 $g.L^{-1}$ loads, respectively. In general, the time needed to reach maximum cumulative hydrogen level increased with the initial glucose concentrations. Noticeably, the time to reach the maximum value of hydrogen accumulation (*i.e*. around 15 days for 10-20 $g.L^{-1}$ in this study) was longer, comparing with 6-8 days on 10-30 $g.L^{-1}$ reported by Argun *et al.* (2008). The buffer selection was probably the main explanation for this difference. In this study MES of 40mM was used as buffer while in that of Argun *et al.* (2008) it was a phosphate buffer. Similarly, as shown in Figure III-1, in same run of trial, a time of 4-5 days was sufficient to reach a maximal hydrogen accumulation with phosphate buffer whereas around 10 days were necessary for those with MES buffer. Moreover, longer time to reach maximum BHP was also due to lack of oligo-elements, as demonstrated in Figure III-5. In addition, as shown in Figure III-6, the amount of cumulative hydrogen volume increased while increasing the initial glucose concentration up to 16 $g.L^{-1}$. A further increase in glucose concentration, *i.e.* 20 $g.L^{-1}$, resulted in lower cumulative hydrogen formation. Noticeably, the cumulated volume of hydrogen produced at 1 $g.L^{-1}$ (*i.e.* 43 mL) was small and might therefore increase the technical error of evaluation.

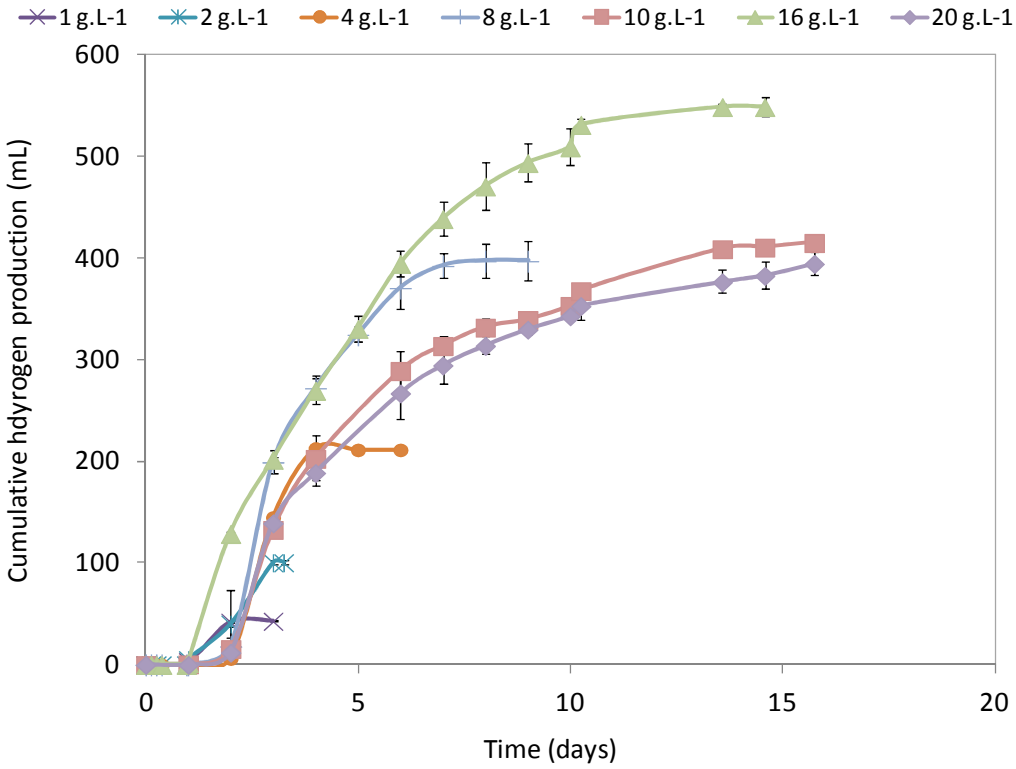


Figure III-6. Cumulative hydrogen production curves (in mL) over the experimental time (in days). The batch tests were operated with an initial pH of 5.5, at 37 ºC, with glucose concentrations ranging from 1 to 20 $g.L^{-1}$.

Furthermore, for initial organic loads higher than 10 $g.L^{-1}$, a metabolic shift occurred obviously from hydrogen forming pathways (*i.e.* acetate-butyrate) to other dissimilative no hydrogen–producing metabolic pathways indicating that the hydrogen-production pathways were overloaded by high substrate load (Figure III-7).

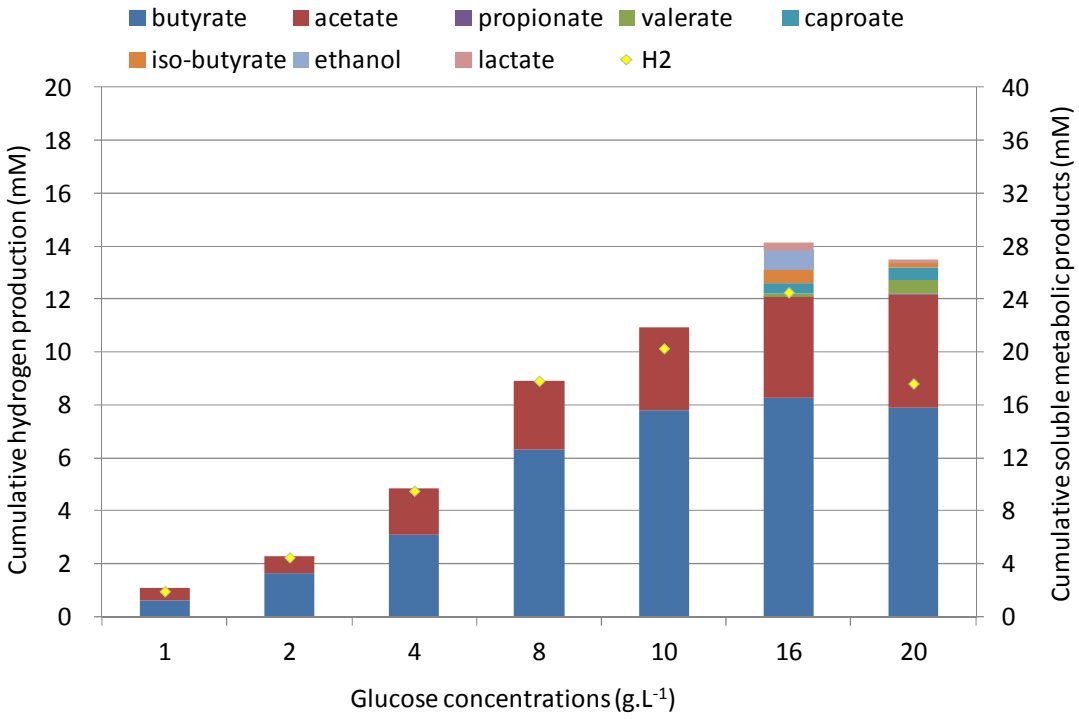


Figure III-7 Metabolites and hydrogen produced by the different cultures at steady state. The Y-Axe is scaled to represent the difference between the H_2 productivity and the associated metabolites produced, both in mM. As an example, if H_2 was only produced by acetate and butyrate pathways, it would be graphically at the same level than the sum of acetate and butyrate (blue + red bars).

Figure III-7 illustrated that below 10 $g.L^{-1}$ of organic load, acetate and butyrate were the sole end-products while at 16 and 20 $g.L^{-1}$ of organic load, other metabolites such as propionate, caproate, valerate, ethanol and lactate were generated.

The stoechiometric and theoretical assumption of hydrogen production from glucose was calculated according to the final acetic and butyric acid contents with the Equation III-1 and Equation III-2 as following:

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{(III-1)}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \text{(III-2)}$$

Based on Equations (III-1) and (III-2), the molar ratio of H_2 generated and butyrate or acetate concomitant is always 1:2. In other words, if the glucose added is totally converted to acetic or/and butyric acid, the theoretical molar cumulative hydrogen production should be twice the sum of molar concentrations of acetic and

butyric acids. In Figure III-7, the maximal hydrogen production is presented according to the molar concentration of metabolic end-products and is linearly correlated to the acetic and butyric acid accumulation for the tests ranging from 1 $g.L^{-1}$ to 16 $g.L^{-1}$. At 20 $g_{glucose}.L^{-1}$, hydrogen production (in mmol) was much less than twice of the sum of butyrate and acetate (in mmol) indicating that acetate was over-produced likely from homoacetogenesis.

Figure III-8 shows the hydrogen yields and the pH values, assessed both at the maximum of the cumulative hydrogen curve, and reported to the initial organic load. The hydrogen yields increased concomitantly with increasing initial glucose concentration up to 4 $g.L^{-1}$ and then decreased for higher glucose inlet concentrations with an optimal range of 4–10 $g.L^{-1}$. When organic load (*i.e.* glucose) increased, more VFAs were generated, as a consequence, pH values at the maximum cumulative hydrogen production were lower. But at high organic load (*i.e.* 10, 16 and 20 $g.L^{-1}$) pH values stopped around 3.6. This final pH values plateau from high organic load revealed a pH inhibition for higher organic load. Quéméneur *et al.* (2010) demonstrated that when pH was below 4.0, the activity of hydrogen producing bacteria was substantially limited. This seemed to be a critical value of pH at 4.0, since increasing residual glucose, *i.e.* 0.3 mM, 2.1 mM, 6.1 mM were detected in batch reactors operated with 10, 16 and 20 $g.L^{-1}$of initial glucose, respectively. In the duplicates at 10 $g.L^{-1}$, only one test showed the residual glucose of 0.3 mM. The organic load of 10 $g.L^{-1}$ corresponded thus to the maximum value within the optimal range (2-10 $g.L^{-1}$) of organic loads considering the organic conversion efficiency (Figure III-8).

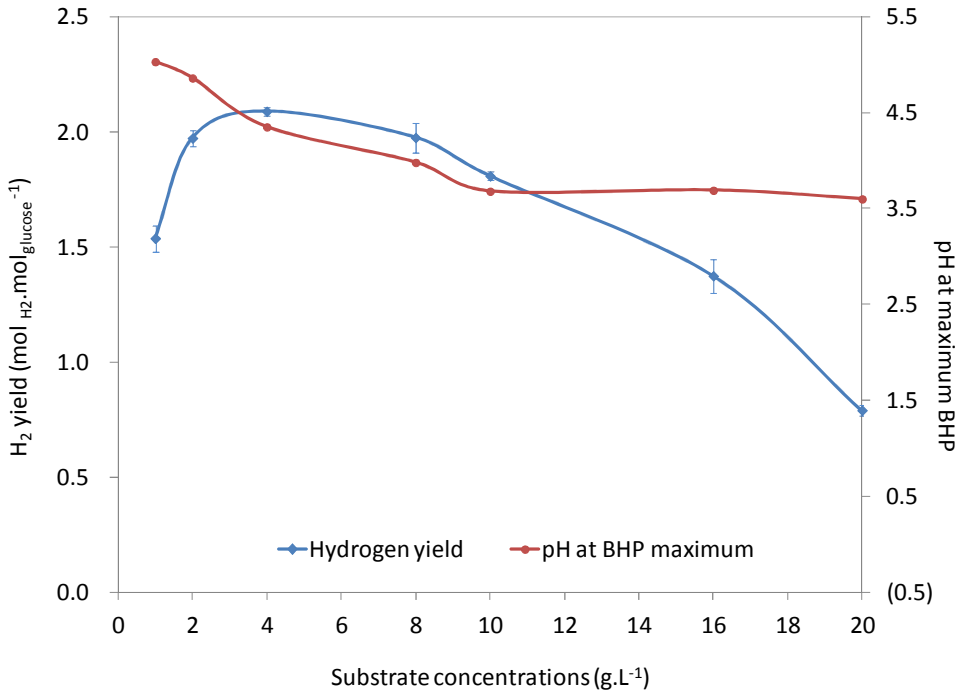


Figure III-8.Variation of hydrogen yield (mol $_{H2}.g_{glucose}^{-1}$) with the initial glucose concentrations ($g.L^{-1}$).

The decreased pH due to accumulation of VFAs was also linked with the change of metabolic pathways (Figure III-7). It is well known that, when pH is lower than 4.5, *Clostridium* sp. shift their metabolic pathways from acidogenesis to solventogenesis (Jones and Woods, 1986; Ginkel *et al.*, 2001; Ferchichi *et al.*, 2005). In this series of test, 2.69 % COD of ethanol was detected in the test at 16 $g.L^{-1}$ (Table III-1). Solventogenesis seemed to result from the accumulation of butyrate and acetate, which influenced the protons transfer through the bacterial cells, as previsouly suggested (Ginkel and Logan, 2005; Zheng and Yu, 2005).

Table III-1 presents the patterns of end-products from different initial organic loads and the mass balance based on COD. The acetic acid and butyric acids were the main VFAs measured compounds for the experiments from 1 $g.L^{-1}$ to 20 $g.L^{-1}$ representing 36 % to 70 % of inlet COD. This indicated that the main type of hydrogen-producing fermentative pathways was acetic-butyric acid fermentation. Our observations are in accordance with other studies previously published (Khanal *et al.*, 2004; Davila-Vazquez *et al.*, 2008). They reported that the fermentation type depended on the inoculum pre-treatment (Ren *et al.*, 2008) or on pH (Khanal *et al.*,

2004). Similarly to our study, the heat-shock pretreatment is commonly considered as the most suitable method to enrich hydrogen producing bacteria and eliminate non-spore forming bacteria such as methanogens (Mu *et al.*, 2007; Wang and Wan, 2008; O-Thong *et al.*, 2009). After treatment, the spore-forming bacteria are mainly belonging to the genus *Clostridium* sp. gathering strict anaerobic bacteria (Ren *et al.*, 2008). The *Clostridium* sp. genus contains mainly hydrogen-producing species (*e.g. Clostridium butyricum; Clostridium acetobutylicum*), but some hydrogen-consuming bacteria as well such as the homoacetogens (*Clostridium aceticum; Clostridium glycolicum*).

COD recovery was in the range from 67 % to 87 %. The lowest COD recovery value occurred at 1 $g.L^{-1}$, indicating the problem of low hydrogen volume evaluation at low organic load. Sufficient initial organic load therefore, should be added in the experimental condition. Here, an initial organic load from 4 to 10 $g_{glucose\_equivalent}.L^{-1}$ was finally recommended. At 4 $g_{glucose}.L^{-1}$, COD recovery value was the highest, as well as the hydrogen yield. Glucose residues represented only 3 % at 10 $g.L^{-1}$, but 12 % and 28 % of inlet COD at 16 and 20 $g.L^{-1}$, respectively, indicating that glucose conversion efficiency decreased when organic load was over 10 $g.L^{-1}$ likely due to an inhibition of the microbial activity by pH or accumulated VFAs. Residual glucose and other metabolic products besides of acetate and butyrate (9 % and 6 % of inlet COD at 16 and 20 $g.L^{-1}$, respectively) also suggested an overloading from the organic substrate. Thus, the optimal range of organic load was estimated in the range of 4 to 10 $g.L^{-1}$.

Table III-1. Metabolic products at the end of the batch and COD mass balance from different initial glucose concentration.

Initial glucose concentration (g/L)	outlet COD*(%)										
	Glucose	Butyrate	Acetate	Propionate	Valerate	Caproate	Iso-butyrate	Ethanol	Lactate	H_2	COD recovery
1	0	41	14	0	0	0	0	0	0	13	67
2	0	60	10	0	0	0	0	0	0	16	86
4	0	57	13	0	0	0	0	0	0	17	87
8	0	58	10	0	0	0	0	0	0	16	84
10	3	58	9	0	0	0	0	0	0	15	86
16	12	39	7	0.1	0.9	3	2	2	0.8	11	78
20	27	30	6	0.2	2.4	3	1	0	0.3	7	76

* The calculation based on data at maximum of hydrogen accumulation points.

III.1.1.5 **Conclusion**

Several trials were carried out to optimize the protocol of biohydrogen potential. MES was selected as buffer to maintain acid-fermentation environment with an optimal buffer concentration at 40mM. Higher MES than 40mM maintained the pH to a higher value, but favoured at the same time the H_2-consuming homoacetogenic bacteria, leading finally to lower hydrogen yields. Oligo-elements had a significant effect on kinetics but almost no effect on hydrogen yield. A range of 4-10 $g.L^{-1}$ was suggested for initial organic load. If organic load was too low, the volume of hydrogen produced can be underestimated, whereas too high organic loads led to lower substrate conversion efficiencies likely due to end-product accumulation. Moreover, and whatever the conditions tested, it was shown that the BHP test resulted from a combination of H_2-producing bacteria and H_2-consuming or H_2-dissimilative pathways. Too high MES buffer concentration as well as too high organic load may favour no hydrogen-producing pathways and this should be considered to further experiments. Therefore, the determination of the optimal organic load results from a fine compromise between the capacity of the microbial consortium to convert efficiently a maximum of organic substrate through hydrogen-producing pathways, and the accumulation of end-products from organic substrate overload that preclude the occurrence of hydrogen-producing pathways. At this stage, a BHP protocol was established and further used to evaluate the possibility of producing hydrogen from various organic substrates.

III.1.2 **BHP determination from organic solid substrates of various compositions and structures**

In this section, diverse organic solid substrates were collected and tested in batch reactor operated according to the BHP protocol previously optimized (see this chapter section III-1.1). The present investigations focused on organic solid waste because among the potential and available substrates, organic solid waste represents an abundant and cheap source of highly biodegradable substrates. Indeed, more than 374 million tons of agricultural and forestry waste are produced in France which represents 43 % of the total annual production of waste (Dubois, 2006). Worldwide, the lignocellulosic biomass residues are evaluated annually about 200 billion tons (Ren *et al.*, 2009). Over the last decades, many studies investigated the possibility to convert different types of waste into biohydrogen. In particular, food waste showed the relatively best performance of conversion into hydrogen. A range of 2.68-8.75 $mmol_{H2}.g_{VS}^{-1}$ was reported for food waste collected in restaurants (Kim *et al.*, 2004; Lay *et al.*, 2005; Shin and Youn, 2005; Li *et al.*, 2008). Nevertheless, behind the term of "food waste" that was investigated as feedstock for hydrogen production, a wide variety of substrates is represented including kitchen refuse, municipal waste, food industry co-products such as oil mill, cheese whey and starch-manufacturing waste (Guo *et al.*, 2010). In addition agri-industrial waste represents a very promising feedstock, and has been also widely investigated. With this type of substrate, high biohydrogen production yields were obtained with 3.77 $mmol_{H2}.g_{VS}^{-1}$, 11.67 $mmol_{H2}.g_{hexose}^{-1}$ and 12.95 $mmol_{H2}.g_{VS}^{-1}$ for palm oil mill effluent, molasses and cheese whey, respectively (Aceves-Lara *et al.*, 2008; Chang *et al.*, 2008a; O-Thong *et al.*, 2008a; Venetsaneas *et al.*, 2009). The use of waste generated from the primary agricultural sector such as maize stalk or rice straw is usually reported with acidic, enzymatic or microwave pretreatments leading to few data of hydrogen production from raw agricultural waste. Overall, the published hydrogen production potentials vary a lot for a given substrate, mainly because of variable composition and definition of a feedstock, as well as differences in the experimental procedures, *e.g.* in batch or continuous reactors and the origin of the microbial inoculum (Kim *et al.*, 2004; Lay *et al.*, 2005; Li *et al.*, 2008).

Structurally, when organic solid waste is degraded in mixed microbial cultures, it is first decomposed in small polymers or soluble molecules such as carbohydrates, proteins or amino acids and fats. Then, these compounds, and more particularly carbohydrates, are converted to VFAs concomitantly with the production of hydrogen and carbon dioxide. More especially, carbohydrate hydrolysis leads to the release of monomeric units of hexose and pentose. At this stage, *Clostridium* sp. are the main bacteria producing hydrogen by hexose and pentose fermentation. In contrast, about fermentative protein metabolisms, proteins are first hydrolyzed into amino acids and then these latter are catabolized through a Stickland reaction or fermented to form H_2 and CO_2 with volatile fatty acids and NH_4^+ generation (Karlsson *et al.*, 2008). Finally, fats (triglycerides) are hydrolyzed to glycerol and three fatty acid chains through ß-oxidation. Although glycerol can be converted to hydrogen and VFAs (Akutsu *et al.*, 2009), the degradation of the fatty acid chains does not generate hydrogen spontaneously since it is rapidly thermodynamically unfavourable at low partial pressure of hydrogen (Schink, 1997; Karlsson *et al.*, 2008). Thus, considering these three main organic categories of structural components found in organic waste, carbohydrate and protein compositions represent the two main fractions potentially involved in hydrogen production pathway, and they require therefore a particular focus.

The purpose of this subchapter is to investigate the impact of the nature and quality of the solid organic waste to the biological hydrogen potentials (BHP), and how the waste type influences the biohydrogen fermentative pathways. A special focus was made on carbohydrate and protein compositions. For this, hydrolysis of organic material was carried out with appropriate NaOH (0.5N) and acid (HCl – 2N) pretreatment for all type of substrates, to determine, respectively, the protein and the main total carbohydrate contents, so-called total carbohydrate$_{HCl}$. A multivariate analysis by partial least square regression technique was then performed to analyze the relationship of substrate compositions and BHP. Moreover, a predictive model of hydrogen production from the biochemical composition of the substrates and the metabolite production from microbial H_2-producing fermentation was proposed after PLS analysis. Finally, a specific method called Van Soest method was carried

out on agricultural waste to finely characterize the different carbohydrates found in lignocellulosic substrates (soluble carbohydrates, so-called carbohydrate$_{NDF}$; and cellulose-like, hemicellulose-like and lignin-like fractions). At this stage, another PLS analysis helped us to understand the relationship between these carbohydrate parts of lignocellulosic substrates and the hydrogen pathways.

III.1.2.1 **Biochemical characterization of organic complex substrates**

In a first series of experiments, 26 organic solid substrates were collected and were investigated in BHP tests. Among them, 21 substrates were analyzed to determine the carbohydrate$_{HCl}$ and protein compositions after solubilization with 2N HCl and 0.5N NaOH and for quantification with anthrone method and Bicinchoninic Acid Protein Assay Kit, respectively (see section II-3.1.2 and II-3.1.3).

The distribution in biochemical components of all the organic substrates is given in Table III-2. The carbohydrates$_{HCl}$ fraction represented from 51 % to 95 % of the total solids and from 70 % to 100 % of the volatile solids for apples, carrots, wheat flour, Jerusalem artichoke tubers, maize flour, and oats. This group was thus referred as carbohydrate-rich substrates. Proteins represented from 14 % to 77 % of total solids and from 69 % to 100 % of volatile solids for chicken meat, cow manure with straw, fish residues, meat residues from restaurant, grape marc, and soybean milk cake. This group was thus considered as protein-rich substrates. The other substrates corresponded to complex organic mixtures and showed a balanced proportion of carbohydrate$_{HCl}$ and protein contents. They were therefore categorized in two groups according to their origins, *i.e*. agricultural or agri-industry waste. Interestingly, since about 72 % and 76 % of total solids of grape marc and soybean milk cake were proteins, these two substrates were rather classified into protein-rich content feedstock than agri-industry waste.

In this study, it is important to notice that the lipid and lignin contents of the substrates were not characterized because these two compounds do not represent potentially a major fermentable source for hydrogen production (Kaparaju *et al.*, 2009). Moreover, the monomeric constituents of cellulosic polymers are hard to

solubilize when embedded within the lignocellulosic matter and were therefore not totally extracted by the method used in this study, *i.e.* mild acidic hydrolysis with 2N hydrochloric acid. That could explain the very low content in carbohydrate$_{HCl}$ contents determined in lignocellulosic substrates such as giant reed stalks, maize stalks and rice straws.

Table III-2. Maximum cumulated hydrogen amount (H_{max}), biochemical composition of solid organic substrates (total carbohydrate$_{HCl}$ and protein).

Substrat	H_{max} ($ml_{H2}.g_{TS}^{-1}$)	Carbohydrate$_{HCl}$ ($mg_{Glu}.g_{TS}^{-1}$)	Proteins ($mg_{BSA}.g_{TS}^{-1}$)	Type of substrates
Apple	113	683	290	carbohydrate-rich
Carrot	1373	513	0	carbohydrate-rich
Wheat flour	147	834	0	carbohydrate-rich
Jerusalem artichoke tuber	120	772	13	carbohydrate-rich
Maize flour	224	952	3	carbohydrate-rich
Oat	169	727	3	carbohydrate-rich
Potato	173	nd	nd	carbohydrate-rich
Soybean milk cake	16	7	765	protein-rich
Chicken meat	7	0	190	protein-rich
Cow manure with straw	3	88	192	protein-rich
Fish residues	2	1	143	protein-rich
Meat residues from restaurant	6	40	151	protein-rich
Grape marc	1	61	720	protein-rich
Food waste from restaurant	71	230	100	agri-industry waste
Fruit peels	13	289	11	agri-industry waste
Maize cob	62	243	76	agri-industry waste
Rapeseed oil cake	43	103	97	agri-industry waste
Sunflower oil cake	16	69	68	agri-industry waste
Vegetable residues from restaurant	46	nd	nd	agri-industry waste
Giant reed leaf	25	138	51	agricultural waste
Giant reed stalk	34	69	34	agricultural waste
Jerusalem artichoke leaf	15	nd	nd	agricultural waste
Jerusalem artichoke stalk	69	293	0	agricultural waste
Maize stalk	4	nd	nd	agricultural waste
Rice straw	24	152	33	agricultural waste

Sorghum	35	nd	nd	agricultural waste

III.1.2.2 **Biohydrogen production potentials from the organic solid substrates**

The biogas produced in all the batch cultures for all the 26 tested substrates was only composed of H_2 and CO_2 along with the fermentation process, with no detectable CH_4. This result shows that the heat-shock pretreatment applied on the inoculum was efficient in suppressing the methanogenic activity. The coefficients R^2 determined by fitting the Gompertz curve for all BHP experiments were all over 0.98, indicating that this model was suitable to describe the kinetics of the BHP tests. Figure III-9 represents few examples of the kinetics of each type of substrate, with the determination of maximum accumulated hydrogen production values, so-called biohydrogen potentials or H_{max}. The average H_{max} values of each substrate are plotted in Figure III-10, including the minimal and maximal values of the duplicates. Glucose was used as control in each experiment series to test the efficacy of the inoculum, giving Hmax of 229±22 $mL_{H2}.g_{glucose\ added}^{-1}$. An analysis of variance (one-way ANOVA) on Hmax of organic substrates tested clearly shows that the substrate category had a highly significant effect on hydrogen production performances (p-value<0.001). The average H_{max} values were, in decreasing order 155 ±38 $mL_{H2}.g_{TS}^{-1}$, 42 ± 24 $mL_{H2}.gTS^{-1}$, 29 ± 20 $mL_{H2}.g_{TS}^{-1}$ and 6 ± 5 $mL_{H2}.g_{TS}^{-1}$ for carbohydrate-rich-content substrates, agri-industry waste, agricultural waste and protein-rich-content substrates, respectively. In conclusion, the carbohydrate-rich-content substrates represented the most favorable feedstock for hydrogen production by dark fermentation.

Indeed, carbohydrate-rich substrates have been referred in the literature to be the main support of biohydrogen production through fermentative pathways, and our results are consistent with this assumption (Hawkes *et al.*, 2002). The metabolic pathways associated to carbohydrate catabolism play a main role in hydrogen generation, and particularly through the acetate and butyrate pathways (Li and Fang, 2007). This explains why carbohydrate-rich substrate favored hydrogen production by dark fermentation. It is worth to note that maize flour exhibited the highest BHP value (224 ± 4 $mL_{H2}.g_{TS}^{-1}$), which is close to the theoretical value *i.e.* 311 $mL_{H2}.g_{hexose}^{-1}$, proposed by Hawkes *et al.* (2007) who suggested a theoretical and average value of hydrogen yield of 2.5 $mol_{H2}.g_{hexose}^{-1}$ in mixed culture.

In contrast, a range of 2-7 $mL_{H2}.g_{TS}^{-1}$ was observed for the protein-rich substrates, showing that the protein composition was not a favoring factor for biohydrogen production. In the literature, the hydrogen yields from protein-rich substrate are globally low, *e.g.* 7 $mL_{H2}.g_{TS}^{-1}$, 8 $mL_{H2}.g_{TS}^{-1}$ and 21 $mL_{H2}.g_{TS}^{-1}$ from eggs, lean meat and bean curd manufacturing waste, respectively (Noike *et al.*, 2000; Okamoto *et al.*, 2000). Such conclusion on the inappropriate use of substrates rich in proteins to hydrogen conversion was also confirmed by the protein metabolism studies in anaerobic conditions: proteins are indeed first hydrolyzed into amino acids and their subsequent degradation shift to either the Stickland reaction or fermentation pathways to form H_2 and CO_2 with volatile fatty acids and NH_4^+ production. The Stickland reaction is a redox-reaction with no hydrogen production where one amino acid is used as electron donor and another amino acid acts as electron acceptor to form a carboxylic acid (*e.g.* acetate) and CO_2. This reaction seems to be more energy favorable and does not require low hydrogen partial pressure to occur. It is likely dominant for protein degradation in comparison with the second alternative pathway of fermentation, especially in presence of clostridia species (Karlsson *et al.*, 2008). This is consistent with our results where protein-rich substrates were not favorable to hydrogen production.

In the group of agri-industry waste and agricultural waste, several substrates showed promising hydrogen production potentials. The sunflower and rapeseed oil cakes are the main waste from oil industry with the advantage to be easily dried and pressed. They presented interesting hydrogen potentials of 16 ± 2 $mL_{H2}.g_{TS}^{-1}$ and 43.3 ± 0.9 $mL_{H2}.g_{TS}^{-1}$, respectively. Other interesting potential substrates revealed in our study were food waste including vegetable waste and stalks like giant reed stalks, sorghum and Jerusalem artichoke stalk.

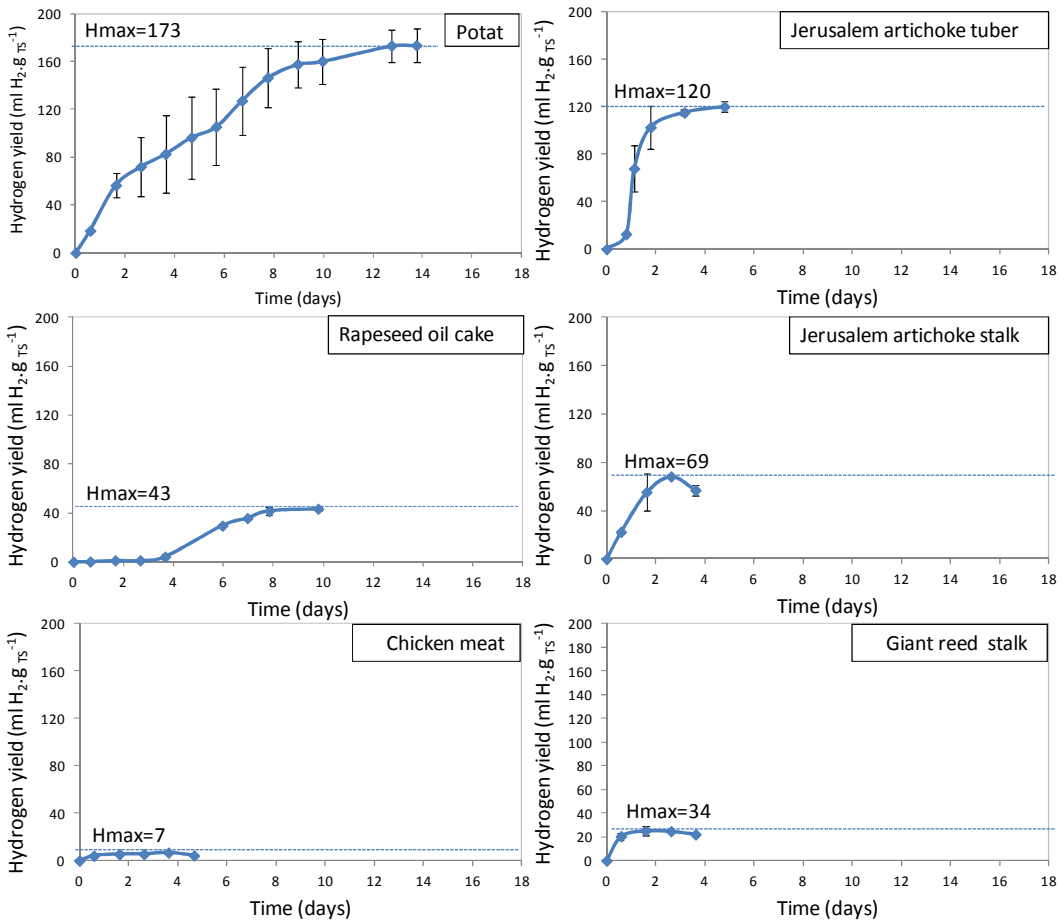


Figure III-9. Examples of hydrogen production kinetics of organic solid substrates in batch tests and determination of the maximum cumulated hydrogen production, H_{max}, corresponding to "biohydrogen potential" of the corresponding substrate.

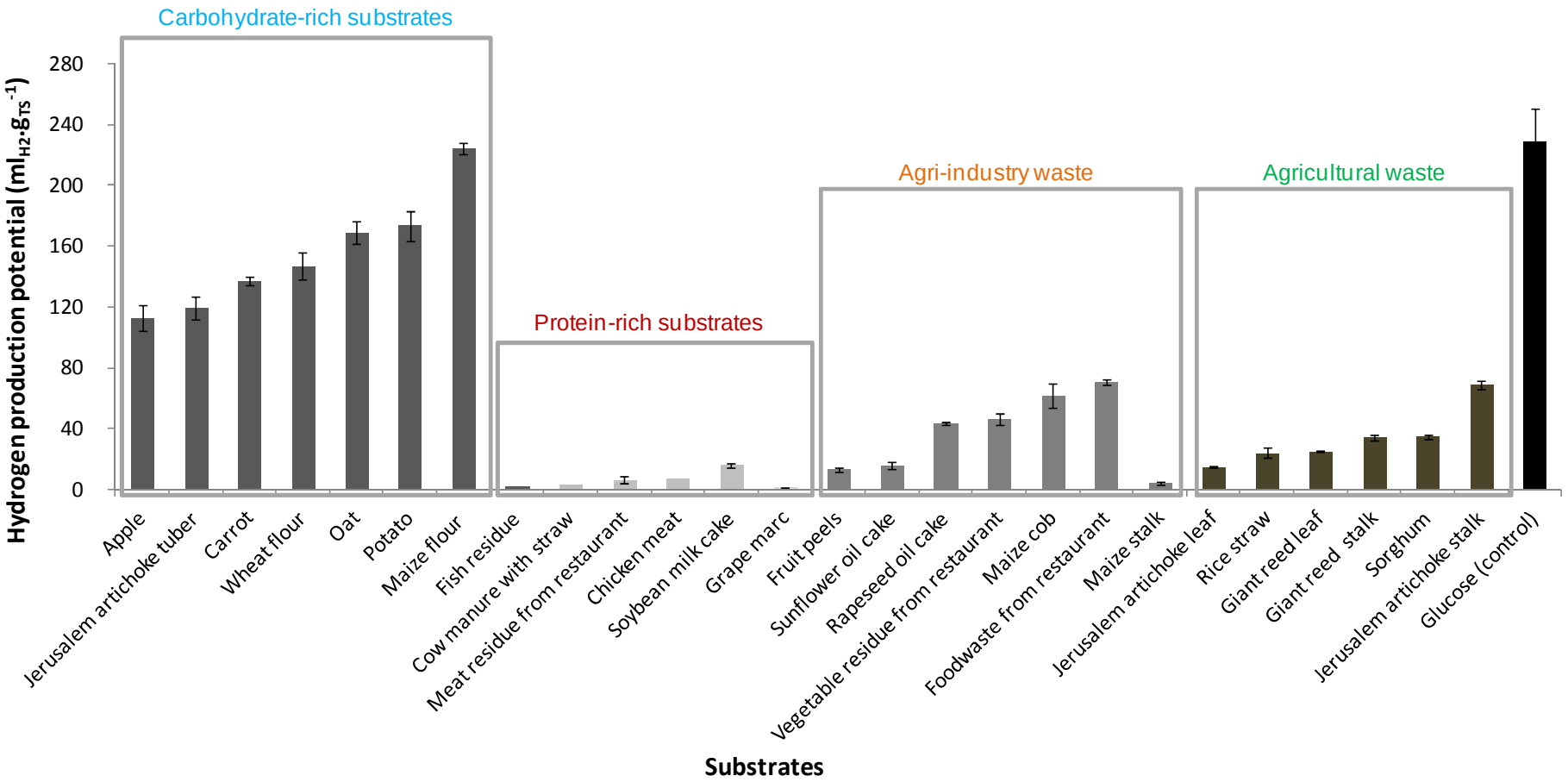


Figure III-10. Biohydrogen production in BHP tests of various organic solid substrates. Considering their characterization and origins, the substrates are classified in four categories: carbohydrate-rich, protein-rich, agri-industrial and agricultural waste. Glucose was used as witnesses in each run of trials.

III.1.2.3 **End-products produced by different type of substrates**

All the fermentative end-product concentrations at the maximum of biohydrogen production (H_{max}) are presented in Table III-3. In this study, acetate and butyrate were the two main end-products besides hydrogen and carbon dioxide. Except for few substrates, lactate and propionate were detected at low concentration compared to the sum of total volatile fatty acids. Acetate and butyrate were the main soluble metabolic products indicating that the metabolic network concerned mainly the hydrogen-producing pathways of acetate-butyrate fermentation type. This was probably due to the thermal pre-treatment of the inoculum which led to a strong selection of spore-forming bacteria, *i.e*. mainly *Clostridium* species (Guo *et al.*, 2010). Moreover, butyrate and acetate are primary soluble metabolites generated by *Clostridium* sp. at pH 5.5 and mesophilic temperature of 37 °C (Lin *et al.*, 2007; Ren *et al.*, 2008).

High concentrations of lactate were detected in the tests on potato and fruit peel, and smaller but significant amounts of lactate were observed in tests with others substrates such as Jerusalem artichoke tubers, giant reed stalk and leaves (Table III-3). No lactate accumulated in the tests with protein-rich substrates, suggesting that lactate production should be related to carbohydrate metabolism. Lactate production could be the result of a metabolic shift either within one microbial population or from the change in bacterial species within the mixed culture. Lin *et al*. (2007) showed that when glucose was the limiting source of carbon, *Clostridium butyricum* shifted from acetate/butyrate to lactate/ethanol production, and even when glucose was additionally supplied, the metabolic shift did not return to the acetate/butyrate hydrogen-producing pathways. Otherwise, in mixed cultures, some other bacteria resistant to heat treatment, such as *Bacillus racemilacticus,* could outcompete with clostridial hydrogen producers to use carbohydrates and form lactate (Hyronimus *et al.*, 2000).

Propionate was found in small amounts for all type of substrates. The highest concentration of propionate was obtained with the tests on Jerusalem artichoke leaves. Propionate apparently resulted from the presence of specific microorganisms

such as *Propionibacterium* species, which are able to use carbohydrate-base feedstocks or glycerol as carbon sources (Himmi *et al.*, 2000). Propionate production is reported to be higher when using glycerol rather than glucose as substrate in fermentative conditions (Zhang and Yang, 2009). This previous observations are consistent with our study where propionate concentrations were high in the tests fed with probable fatty substrates, *i.e*. sunflower oil cakes, rapeseed oil cakes, meat residue from restaurant, chicken meat and fish residues (Table III-3).

Table III-3. Maximum hydrogen production (H_{max}) and end-product production (acetate, butyrate, propionate and lactate) when the maximum hydrogen production was reached.

Substrat	H_{max} ($ml_{H2}.g_{TS}^{-1}$)	Acetate at H_{max}time ($mg.g_{TS}^{-1}$)	Butyrate at H_{max}time ($mg.g_{TS}^{-1}$)	Propionate at H_{max}time ($mg.g_{TS}^{-1}$)	Lactate at H_{max}time ($mg.g_{TS}^{-1}$)
Apple	113	96	88	11	0
Carrot	137	189	289	9	24
Wheat Flour	147	148	149	0	16
Jerusalem artichoke tuber	120	18	176	0	32
Maize flour	224	158	336	6	0
Oat	169	85	223	2	12
Potato	173	126	151	2	189
Soybean milk cake	16	111	69	3	0
Chicken meat	7	89	38	6	0
Cow manure with straw	3	9	5	0	0
Fish residue	2	177	20	14	0
Meat residue from restaurant	6	75	44	3	0
Grape marc	1	9	0	0	0
Foodwaste from restaurant	71	55	71	1	0
Fruit peels	13	28	2	0	286
Maize cob	62	55	106	0	48
Rapeseed oil cake	43	15	67	4	0
Sunflower oil cake	16	36	41	1	0
Vegetable residue from restaurant	46	60	77	2	0
Giant reed leaf	25	11	13	0	6
Giant reed stalk	34	24	19	0	14
Jerusalem artichoke leaf	15	207	141	35	0
Jerusalem artichoke stalk	69	55	109	0	20
Maize stalk	4	nd	nd	nd	nd
Rice straw	24	8	5	0	4
Sorghum	35	nd	nd	nd	nd

nd : not determined.

III.1.2.4 **Substrate mapping considering biochemical composition, end-products and H_{max}**

A PLS (Partial Least Square) regression was carried out to map the substrates from the biochemical and end-product composition oriented to hydrogen production. Indeed, the biohydrogen potential values (BHP), expressed as H_{max}, of 21 substrates

were used as the Y-block variable and six explicative variables as the X-block: the biochemical fractions defined by the $carbohydrate_{HCl}$ and total proteins compositions of the substrate before fermentation, and by the end-product concentrations such as acetate, butyrate, propionate and lactate, all determined at the end of fermentation. Figure III-11A and Figure III-12A present the projection of 21 substrates in the plans formed by the three first principal components or latent variables. The three first latent variables t1, t2 and t3 represented 41 %, 21 % and 19 % of the total variance of the X-block variables, respectively, for a total variance of 81 %. All the carbohydrate-rich substrates were located on the right side of the figures and the other substrates on the left indicating high hydrogen production values for the carbohydrate-rich substrates. The t2 latent variable discriminated the protein-rich substrates with low values on t2 for agricultural substrates, and with high values on t2 for fish substrate (Figure III-11A). In addition, agricultural substrates exhibited low values on t3 (Figure III-12A). Finally, agri-industry waste was the most spread group with high and low values on t3 (Figure III-12A).

Figure III-11B and Figure III-12B present the correlation circles of the original variables (X-block and Y-block variables) with the three first latent variables. By convention, the Y variable (BHP) was located on the right following the first latent variable indicating that the variability of the BHP value can be well explained by the PLS model. The correlation circle showed that the $carbohydrate_{HCl}$ content of the substrate as well as the butyrate concentration determined at the maximal BHP time correlated well with the BHP values. Moreover, acetate and propionate productions were close to the t2 latent variable and did not really correlate with hydrogen production (Figure III-11B). The proximity of acetate and propionate productions might be explained by the propionate metabolism related to acetate accumulation from the presence and the activity of *Propionibacterium* sp. on lactate intermediate, as previously suggested by Vadlani *et al.* (2008). Lactate and protein concentrations explain the t3 latent variable (Figure III-12B) and are oppositely correlated. Because all the latent variables were independent and orthogonal between them, the lactate production could be regarded as an independent variable without any correlation with hydrogen production.

From a biochemical point of view, the pathway from glucose to acetate gives the highest yield of hydrogen with 4 molH_2.mol$_{glucose}^{-1}$ and the pathway of butyrate led to a lower yield of 2 molH_2.mol$_{glucose}^{-1}$. This suggests that higher concentration of acetate would automatically be associated to higher hydrogen production. However, our results showed that acetate production was not a good indicator of hydrogen yield. This could be explained by the occurrence of homoacetogenesis concomitantly to biohydrogen production where hydrogen and carbon dioxide were consumed to produce acetate (Siriwongrumgson *et al.*, 2007). Therefore acetate was generated either by hydrogen producing bacteria, or by hydrogen consumers, such as homoacetogens. In addition, some spore-forming bacteria acetogens like *C. scatologens*, *C. magnum* or *C. coccoides* could also use carbohydrates to produce acetate without release of hydrogen gas, and outcompete hydrogen producers (Kim *et al.*, 2006).

In contrast, the butyrate pathway is inevitable in anaerobic hydrogen fermentation in mixed culture, and no hydrogen consumption pathway has been observed related to butyrate production. This implies that metabolic butyrate pathway was directly related to hydrogen production, and butyrate accumulation represented a better indicator of hydrogen production in dark fermentation than acetate.

As explained above, lactate accumulation resulted from a metabolic shift of *Clostridium* species or was directly produced from carbohydrates by *Bacillus* species. As shown in Figure III-12B, it seems that protein-rich substrates were not favorable to lactate pathways, since a negative correlation between lactate and protein concentrations was observed.

Moreover, acetate and propionate seeemed to be together in the three latent variables. This may be explained the hypothesis of appearance of *Propionibacterium* species, which metabolize acetate and propionate as main end-products fermented.

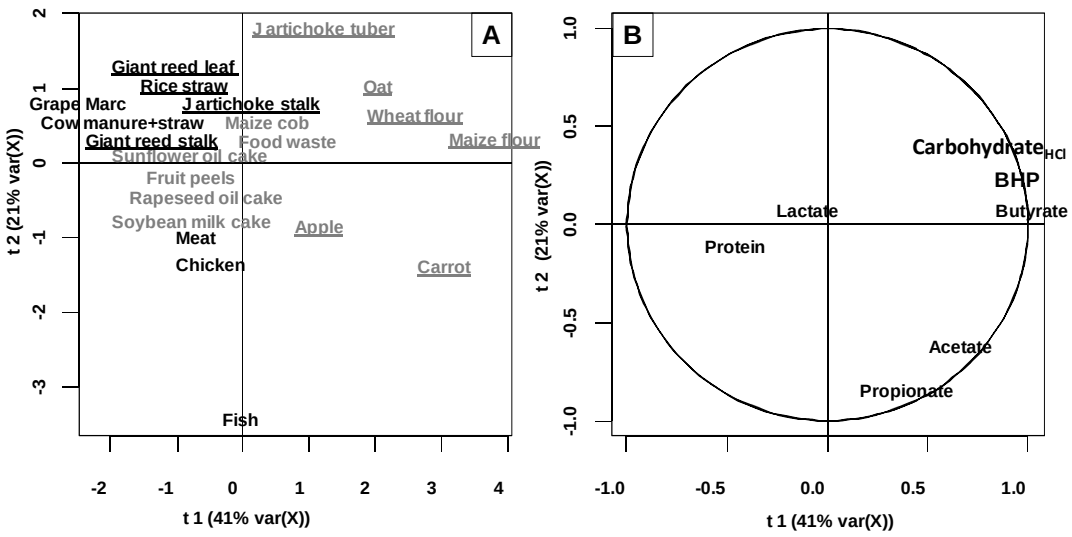


Figure III-11. Principal Component Analysis of the BHP test parameters, carbohydrate and protein composition and end-product accumulation made on 21 different substrates (A): Projection in the two first latent factors (t1, t2) of the 21 substrates. Carbohydrate-rich substrate in grey underlined, protein-rich substrate in black, agri-industrial waste in grey and agricultural waste in black underlined. (B): correlation circle of measured variable. BHP is the predicted variable.

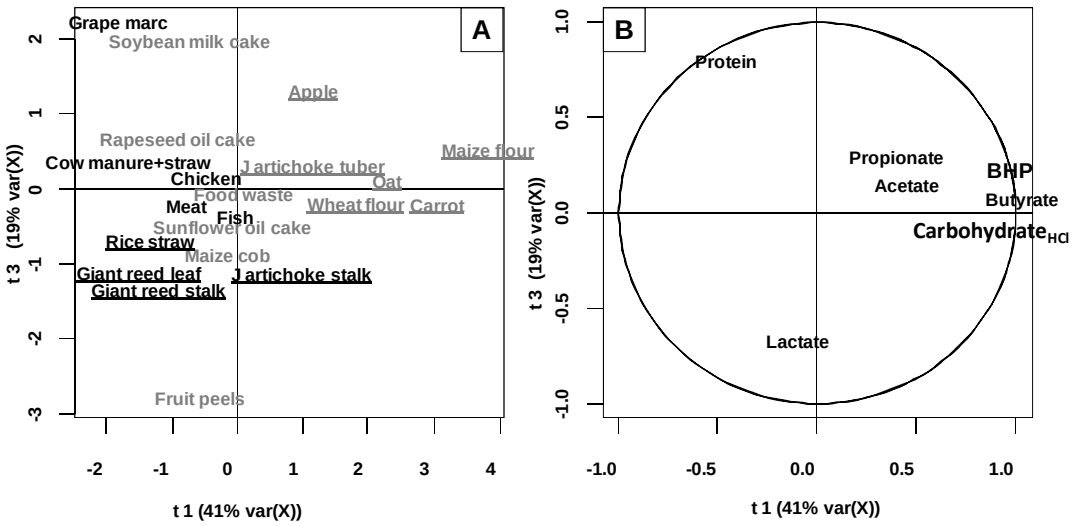


Figure III-12. Principal Component Analysis of the BHP test parameters, carbohydrate and protein composition and end-product accumulation made on 21 different substrates (A): Projection in the first and the third latent factors (t1, t3) of the 21 substrates. Carbohydrate-rich substrate in grey underlined, protein-rich substrate in black, agri-industrial waste in grey and agricultural waste in black underlined. (B): correlation circle of measured variable. BHP is the predicted variable.

III.1.2.5 **Prediction model for biological hydrogen potentials**

PLS analysis is not only able to supply a mapping of the substrates and variables but also can give a predictive model of BHP by calculating its value from experimental values generated from the substrate characterization. In order to achieve good predictive robustness, a one-leave-out cross-validation technique was applied to determine the best number of latent factors which define the model dimension. Figure III-13 plots the value of the PRESS criterion (residual of predicted values) versus the model dimension (number of latent factors). The lowest PRESS value of 0.03 was obtained for a model with three dimensions indicating that three independent sets of variables were required to predict optimally the BHP values. The estimated regression coefficients led to a multi-linear equation given the calculated BHP value following Equation III-3.

$$BHP = 5.149797 + 109.4821*Carb_{HCl} - 19.24518*Prot + 83.51438*Acet + 314.7596*But - 1119.173*Prop - 124.3916*Lact \quad \text{(Eq. III-3)}$$

where $Carb_{HCl}$ and Prot, variables represent $carbohydrate_{HCl}$ and protein contents of the substrates and Acet, But, Prop and Lact variables represent acetate, butyrate, propionate and lactate concentrations at the maximum cumulated hydrogen potential. These variables are expressed in $g.g_{TS}^{-1}$ and BHP values in $mL_{H2}.g_{TS}^{-1}$.

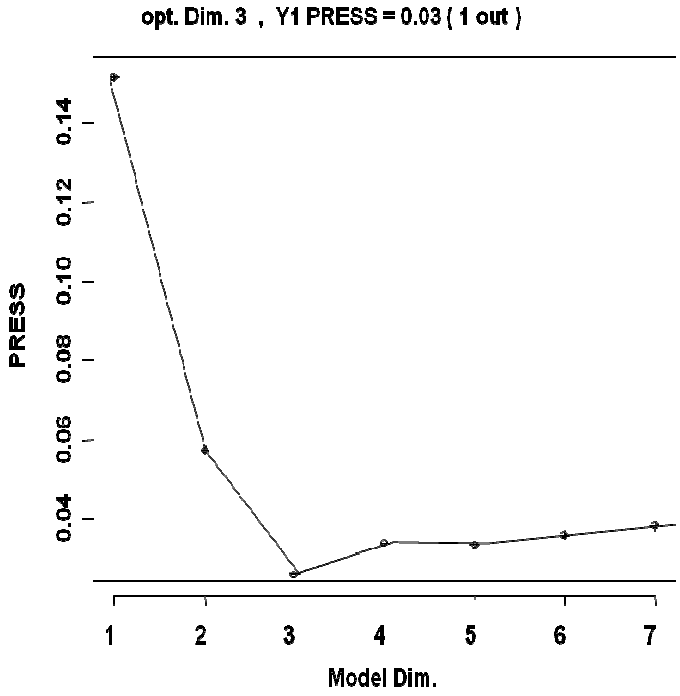


Figure III-13. Predicted residual error sum of square (PRESS) versus number of latent factors (model dimension) of the PLS model with all variables.

Figure III-14 shows the comparison between the hydrogen potentials calculated from substrate composition and metabolite production with Equation III-3. The bisector line is plotted in this graph showing a good repartition of the points around this line. The determination coefficient R^2 is equal to 0.969 confirming the good explanatory capability of this model. The RMSEC/MAX value (the root mean square error of calibration divided by the maximal value) is equal to 14.5/224=0.06 indicating a good accuracy of 6 % of the predictive model.

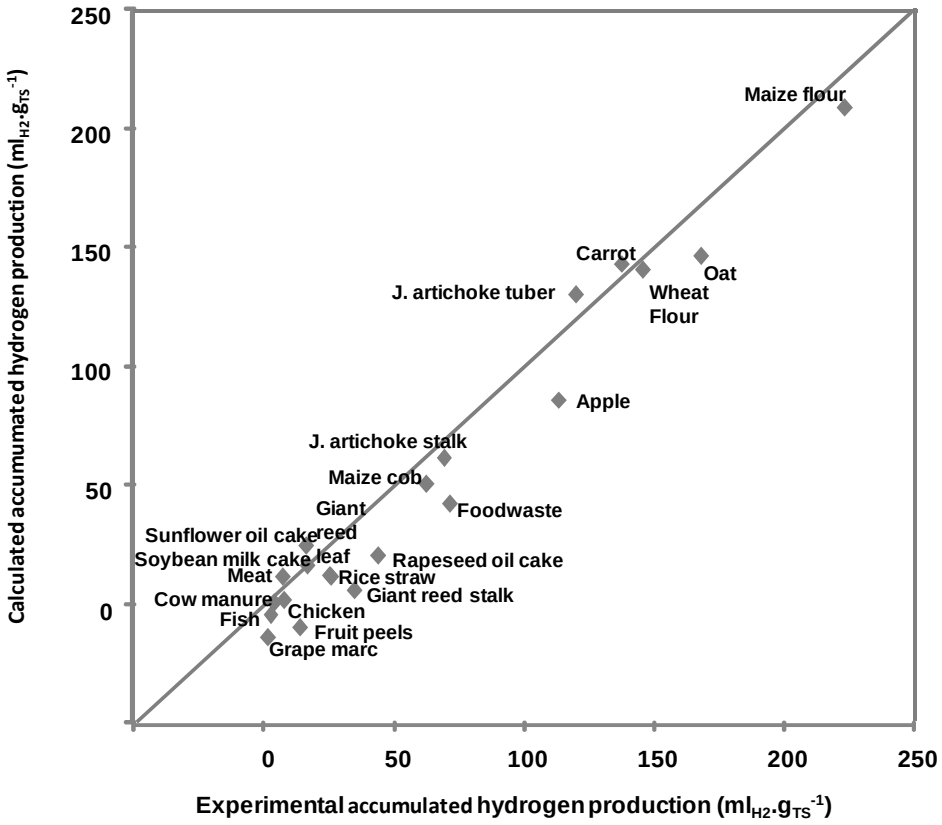


Figure III-14. Calculated versus experimental H_{max} values (maximum cumulated hydrogen production in $mL_{H2}.g_{TS}^{-1}$) of the PLS model based on biochemical characterization and metabolite production. The line represents the bisector.

Figure III-15 shows the regression coefficients sorted by percentage of influence. $Carbohydrate_{HCl}$ and butyrate bars present the highest weights, with relative values of 0.50 and 0.43, indicating that they were the most influential variables on BHP values. Lactate production is the third influential variable with a relative weight of -0.069 showing the negative effect of lactate production on hydrogen production. The standard deviations of the variables were finally calculated by SIMCA (Soft Independent Modeling of Classe Analogy). Although only the standard deviations of carbohydrate and butyrate were satisfied, the regression coefficient weigths of different variables kept the same sesult as showed by R®. Considering that the highest influential variable is the $carbohydrate_{HCl}$ content, a simple linear regression model was established leading to Equation III-4 where the

BHP value is predicted only from the $carbohydrate_{HCl}$ content ($Carb_{HCl}$) expressed in $g.g_{TS}^{-1}$.

$$BHP = 1.3052 + 199.46*Carb_{HCl} \quad \text{(Eq. III-4)}$$

The determination coefficient R^2 of this simple model is equal to 0.89 confirming the good explanatory capability of this model but with a lower value compared to the 0.969 value obtained with the "full" PLS model. Moreover, the RMSEC/MAX value is equal to 21.2/224=0.09 indicating a low accuracy of 9 % of the simple predictive model compared to the accuracy of 6 % determined for the PLS model with all variables. This comparison confirms that $carbohydrate_{HCl}$ content was the main influential variable but butyrate and lactate productions had to be considered to predict with a high accuracy the biological hydrogen production of organic waste.

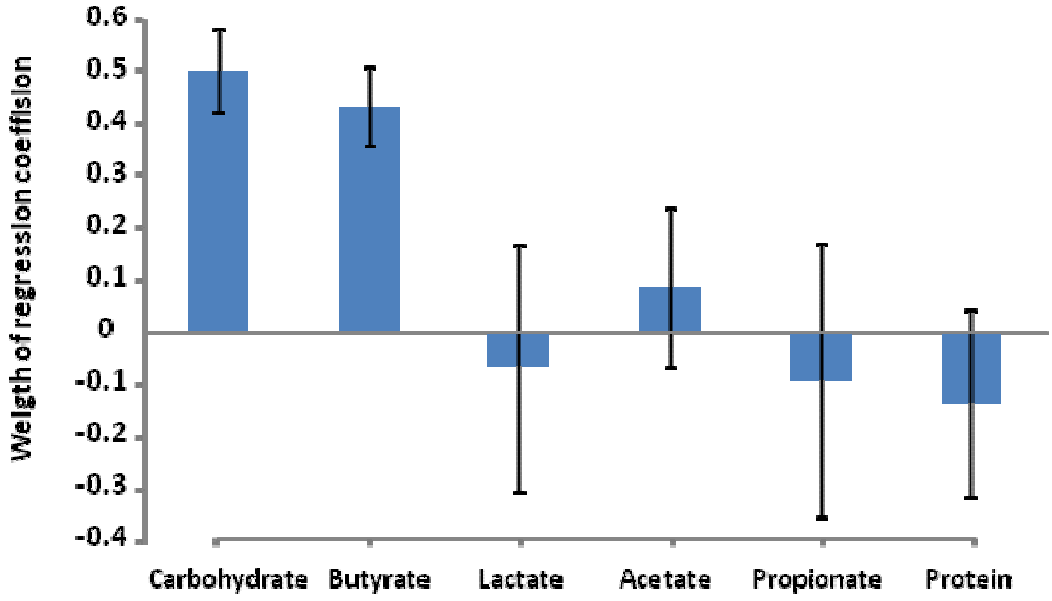


Figure III-15. PLS regression coefficients sorted by percentage of influence.

III.1.2.6 **Fine characterization of carbohydrate composition by fractionation of lignocellulosic substrates and relationship with hydrogen potentials**

As previously shown, agricultural substrates contained $carbohydrate_{HCl}$ contents in a range of 69 to 293 $mg_{glucose\ equivalent}.g_{TS}^{-1}$, based on the acidic hydrolysis with

HCl 2N and further anthrone analysis. This $carbohydrate_{HCl}$ content was directly correlated with hydrogen yields according to PLS analysis (see section III-1.2.4). However, agricultural substrates categorized as lignocellulosic substrates are theoretically composed of mainly polymeric carbohydrates such as hemicelluloses and celluloses. Actually lignocellulosic substrates are composed of three main fractions: hemicelluloses, celluloses and lignin (Monlau *et al.*, 2012): Hemicelluloses are short, highly branched polymers of pentoses (C_5) and hexoses (C_6). The monosaccharides in hemicelluloses are various, including xylose, and arabinose as pentoses, and galactose, glucose, and mannose as hexoses. Comparing to hemicelluloses, the composition of celluloses is simpler: a linear polymer of glucose (C_6). Finally, lignins are amorphous heteropolymers, present in cellular wall. These three fraction of lignocellulosic material are all water non soluble but capable to be solubilized by different detergents (Van Soest, 1963). The method applied to fractionate consecutively these three compositions of lignocellulosic substrate is called "Van Soest" method. In this method, the soluble part is separated by solubilization in a neutral detergent (*i.e.* NDF : neutral detergent fractionation), hemicelluloses in an acid detergent (*i.e.* ADF : acid detergent fractionation), celluloses in H_2SO_4 72 % and the remaining fraction corresponds to lignins (Van Soest, 1963; Dorleans,1998).

In this second series of experiments, seven agricultural substrates (Giant reed leaf and stalk, Jerusalem artichoke leaf and stalk, maize stalk, rice straw and sorghum) and three other substrates (Jerusalem artichoke tuber, sunflower oil cake and maize cob) issued from the carbohydrate-rich and agri-industry waste groups, all potentially containing hemicellulosic or cellulosic components, were fractionated using the Van Soest method. The hydrogen yields as well as the different fractions as defined by the Van Soest method were analyzed by PLS to investigate their relationships. Finally, two complementary experiments were performed to confirm these relationships.

III.1.2.6.1 *Fractionation of organic substrates by the Van Soest method*

Results of the fractionation by the Van Soest method of the ten substrates selected are presented in Figure III-16. The soluble part represented 7 to 73 % of the total solid content. The highest soluble parts were obtained for the whole Jerusalem artichoke plant (tuber, stalk, and leaves) and sunflower oil cake. Hemicelluloses corresponded to 7 to 40 % of these substrates. Cellulose represented 8 to 41 % of the total solid content. Interestingly, cellulose corresponded to the largest fraction in all stalk-based substrates (31 % - 41 % of TS), such as Jerusalem artichoke stalk, maize stalk and giant reed stalk. Significant lignin parts (about 13 % of total solid) were found in sunflower oil cake and giant reed stalk. All the selected substrates exhibited therefore a wide range of fractionating composition, as expected.

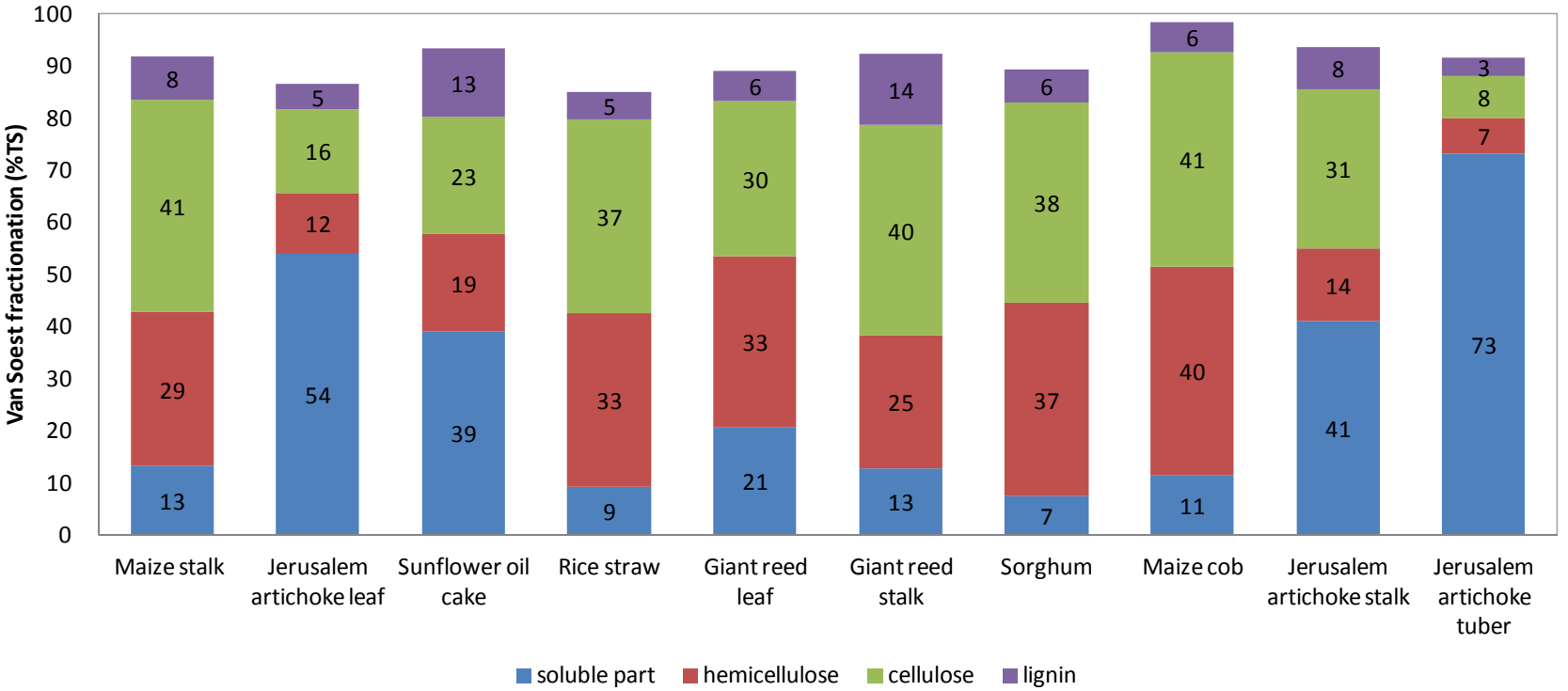


Figure III-16. Van Soest fractionation from ten lignocellulosic substrates.

III.1.2.6.2 *Relationship between Van Soest fractionation and hydrogen yield*

A PLS (Partial Least Square) regression was used to assess whether a correlation existed between the Van Soest fractions and the corresponding biohydrogen yields. Figure III-17B represents the correlation circle with the first two important latent variables, representing 96 % of the variance X. BHP is located on the right part of the circle, but was not placed on the circle, indicating that this model was not very appropriate to predict with accuracy the BHP value. However, being situated on the same side of the correlation circle, BHP values and NDF soluble fractions show a clear positive correlation. This suggests that only the soluble parts might be accessible to anaerobic microorganisms for biohydrogen conversion. Additionally, hemicelluloses and celluloses show negative correlation with BHP. Although some *Clostridium* spp. (*e.g. Clostridium cellulolyticum*) are capable to ferment cellulose (Desvaux *et al.*, 2001), our results showed that the hemicelluloses and cellulose were not utilized for hydrogen production. In other words, these two parts were probably not bioavailable for fermentative hydrogen-producing bacteria. This opposite relationship of cellulose with hydrogen yield was in accordance with previous study performed on methane production. Buffiere *et al.* (2006) reported the sum of the cellulose and lignin fractions of 14 organic substrates presented a linear and negative correlation with the potential of methane productions. The authors suggested the constraints of the microbial bio-availability due to hydrolysis of complex solid substrates. However, in our study, hemicelluloses were not available for biohydrogen production.

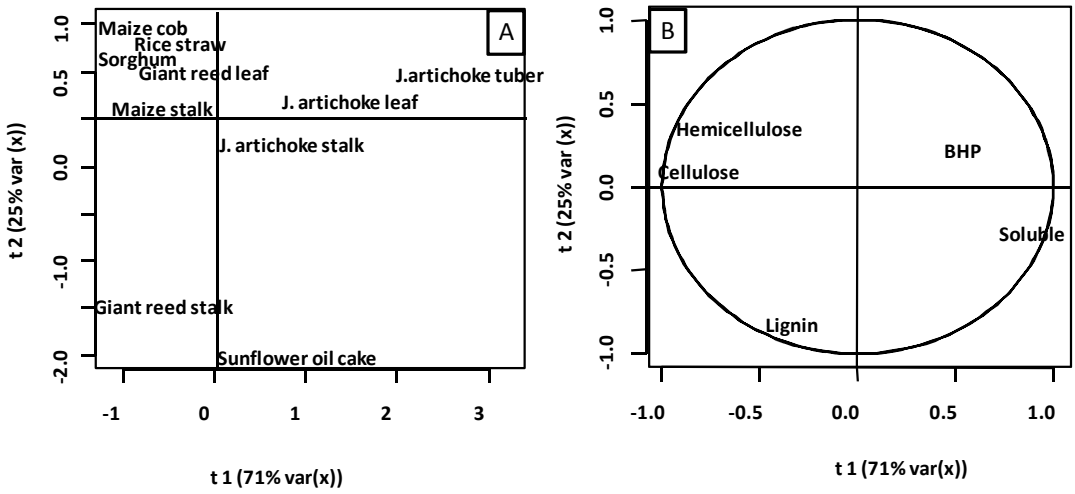


Figure III-17. Principal Component Analysis of the BHP test parameters, carbohydrate and protein composition and end-product accumulation made on 21 different substrates (A) Projection in the two first latent factors (t1, t2) of the ten lignocellulosic substrates. (B) Correlation circle of Van Soest fractionations and the Biohydrogen potential (BHP) variables.

No clear linear correlation between the soluble parts (NDF) and the hydrogen yields was observed (Figure III-18). In NDF, neutral detergent solubilized several organic compounds such as lipids, most of the proteins and soluble carbohydrates$_{NDF}$, and other water-soluble matter (Van Soest, 1967). The positive correlation of soluble parts to hydrogen yields showed in above PLS should be due to some compounds in NDF such as soluble carbohydrate$_{NDF}$. Other parts in NDF (*i.e*. proteins) had no effect on hydrogen yields. In fact, a great proportion of protein content in sunflower oil cake was likely to explain the high soluble part with low hydrogen yields (Raposo *et al.*, 2008). The NDF fraction from Jerusalem artichoke leaves might contain a great quantity of polyphenols as previously reported for olive tree leaves and grape marcs (Amendola *et al.*, 2009; EI and Karakaya, 2009). And the phenolic compounds were also reported as strong inhibitors of fermentative hydrogen production (Quéméneur *et al.*, 2012). A whole characterization of the NDF soluble parts was therefore carried out and is shown in the next section.

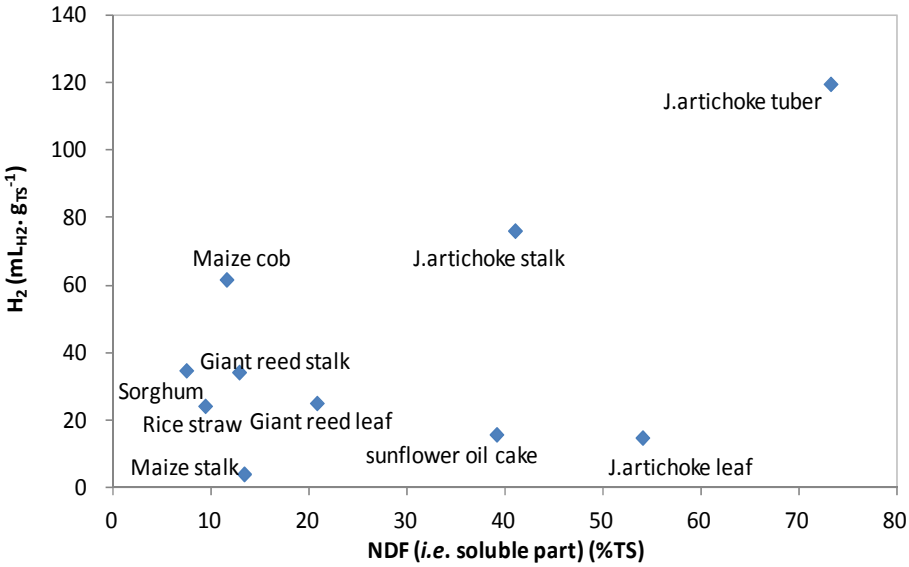


Figure III-18. Distribution of the NDF fraction of ten lignocellulosic substrates obtained by the Van Soest method and related hydrogen yields.

III.1.2.6.3 *Complementary experiment to determine the whole composition of the soluble part with neutral detergent.*

In order to determine soluble carbohydrate$_{NDF}$ and protein contents within the NDF of the lignocellulosic substrates, anthrone method and NTK standard method were applied on these neutral detergent fractions. The results are presented in Table III-4. The significant amount of proteins found in the NDF part of sunflower oil cake confirmed the hypothesis that the protein contents increased the NDF proportion with no relation to hydrogen yield. A linear curve tendency (determination coefficient R^2 of 0.8962) between carbohydrate$_{NDF}$ in NDF and biohydrogen potentials is shown in Figure III-19, confirming previous assumption of soluble carbohydrate available for hydrogen production. Table III-4 shows also that the values of carbohydrate$_{HCl}$, after HCl acid hydrolysis were mainly higher than carbohydrate$_{NDF}$ recovered in NDF, particularly for rice straw, maize cob and giant reed leaves. These three substrates contained mostly hemicellulose (Figure III-16), which is the most thermal-acid

sensitive and easiest to be hydrolyzed for its branched and amorphous structure (Hendriks and Zeeman, 2009). Acid treatment by HCl 2N might have solubilized a small part of hemicelluloses-like compounds within these three substrates.

Table III-4. Comparison of carbohydrate and protein contents measured in two conditions. In the first, $carbohydrates_{HCl}$ and proteins within lignocellulosic substrates were extracted at room temperature by 2N HCl and 0.5N NaOH respectively. In the second, $carbohydrates_{NDF}$ and proteins were solubilized in boiled neutral detergent during one hour.

Substrate	H_2 ($ml_{H2}.g_{TS}^{-1}$)	$Carbohydrates_{HCl}$ after acid hydrolysis ($mg_{Glu}g_{TS}^{-1}$)	$Carbohydrates_{NDF}$ in NDF ($mg_{Glu}g_{TS}^{-1}$)	Proteins after alkaline extraction ($mg_{BSA}g_{TS}^{-1}$)	Proteins in NDF estimated by NTK * ($mg_{BSA}g_{TS}^{-1}$)
Maize stalk	4.1	nd	12	nd	0
Jerusalem artichokes leaf	14.9	nd	50	nd	2
Sunflower oil cake	15.8	69	70	68	24
Rice straw	24.3	152	23	33	0
Giant reed leaf	25.1	138	59	51	0
Giant reed stalk	34.2	69	nd	34	nd
Sorghum	34.8	nd	24	nd	3
Maize cob	61.7	243	167	76	0
Jerusalem artichokes stalk	76.1	293	269	0	0
Jerusalem artichokes tuber	119.5	772	624	13	0

nd: Not determined

* Protein content=NTK * 6.25 (Izhaki, 1993)

The Van Soest method not only confirmed the relationship between $carbohydrate_{HCl}$, $carbohydrate_{NDF}$ and hydrogen yields, but also proved that hemicelluloses and celluloses of the lignocellulosic substrates were not bioavailable for hydrogen production. Furthermore, a good coefficient R^2 of 0.8848 was observed by plotting $carbohydrate_{HCl}$ and hydrogen yields from lignocellulosic substrates (Figure III-20). This is consistent with the PLS model Equation III-3 where the quantity of propionate and lactate were neglected which was the

case for lignocellulosic substrates. Considering the low cost and easy application, HCl 2N hydrolysis and anthrone analysis were recommended as a good method to estimate rapidly the easily solubilized carbohydrate parts and therefore to predict with an acceptable accuracy the hydrogen yield from a defined substrate.

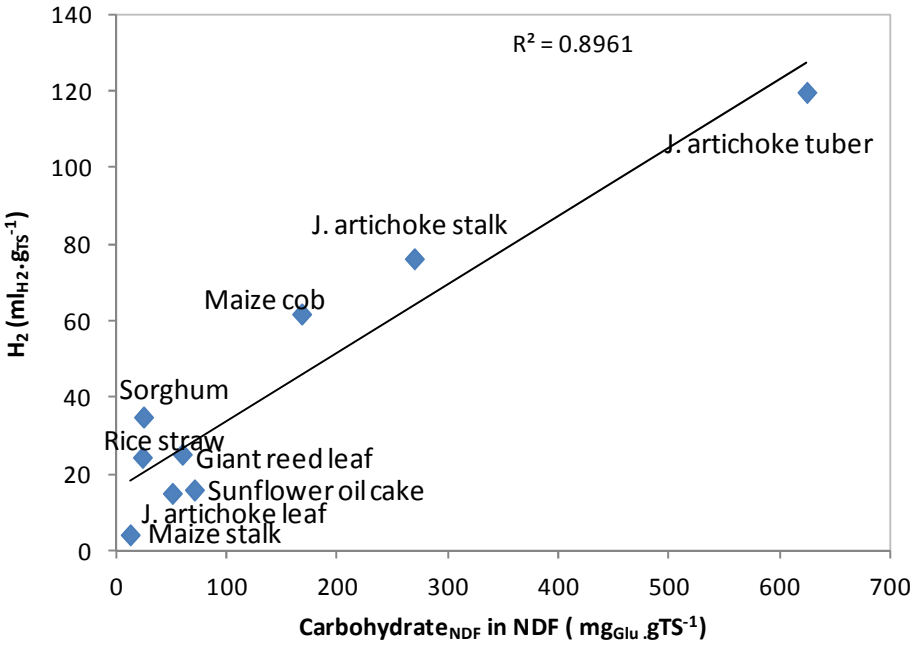


Figure III-19. Relationship between carbohydrate$_{NDF}$ and hydrogen yields of lignocellulosic substrates.

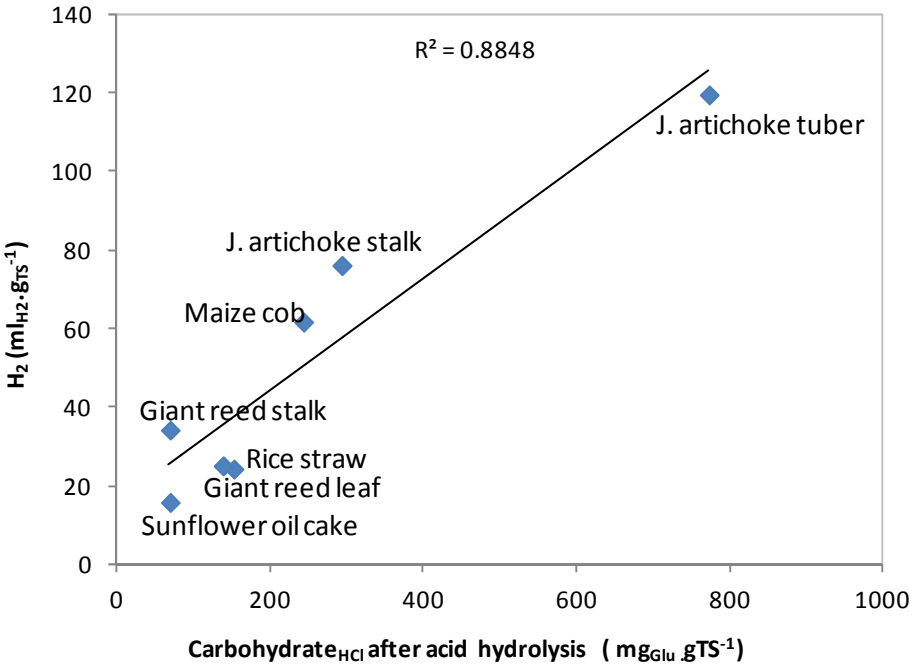


Figure III-20. Relationship between carbohydrate$_{HCl}$ and hydrogen yields of lignocellulosic substrates.

III.1.3 **Conclusion**

This subchapter aimed to investigate the effect of substrates compositions on hydrogen yield and metabolic pathways in mixed culture. In the first section (III-1.1), a protocol for the determination of biohydrogen potential was optimized. An appropriate buffer (MES) at an optimal concentration of 40mM was selected. All the results showed that optimal conditions for hydrogen production resulted from a compromise between control of a suitable range of pH favoring H_2-producing bacteria, and the development of H_2-consuming bacteria growing likely under similar or slightly higher pH (shown when MES concentration was increased). For this reason, the optimal range of organic load was found between 4 to 10 g.L^{-1}. Too high organic load led to the inhibition of end-products due to lower pH and a shift of metabolic pathways from hydrogen producing to dissimilative or H_2-consuming pathways.

In the second section (III-1.2), a large range of organic solid waste was tested with the optimized protocol to assess their biochemical hydrogen potentials (BHP). The impact of the substrate compositions on the hydrogen potentials was evaluated. For this, carbohydrate and protein compositions were determined under mild hydrolysis conditions with HCl (2N) followed by Anthrone quantification and NaOH (0.5N) followed by bicinchoninic acid protein quantification kit, respectively. A statistical one-way ANOVA test showed that the category of substrates had a significant influence on hydrogen potentials with higher hydrogen yields obtained with the carbohydrate-rich substrates. In contrast, low hydrogen yields obtained with the protein-rich substrates, showed that the protein content was unfavorable factor for biohydrogen production. Furthermore, the PLS regression showed that the $carbohydrates_{HCl}$ concentration and the butyrate accumulation strongly correlated with the hydrogen yields observed. A predictive model was thus proposed to estimate with a good accuracy the hydrogen yield (BHP) according to $carbohydrates_{HCl}$ concentrations in organic substrates. Further characterization of ten lignocellulosic substrates was performed using the Van Soest method where the organic materials were fractionated into soluble, hemicellulosic, cellulosic and lignin parts. Another PLS regression analysis showed that only the soluble part of the lignocellulosic substrates positively correlated with the hydrogen yields. Anthrone method applied on neutral detergent fractionates solutions implied linear relationship of soluble $carbohdyrate_{NDF}$ to hydrogen yields. Therefore, it was concluded that the positive correlation of the soluble part to hydrogen yields was mainly due to the content in soluble $carbohdyrates_{NDF}$. In conclusion, the two methods to estimate the carbohydrate contents from lignocellulosic substrates showed that only easily extractable carbohydrates are bio-available for their degradation to hydrogen, but little is known on dynamics of hydrogen production from solid waste in continuous culture, which will be investigated in the next chapter.

III.2 Continuous fermentative hydrogen production and dynamics using a solid substrate: the Jerusalem artichoke tubers

This second part of the study aims to investigate the hydrogen production performances and dynamics in continuous stirred tank reactor systems. For this, the Jerusalem artichoke tubers were selected as solid substrate for their high contents of carbohydrate, *i.e.* fructose based polysaccharides so-called inulin. Characterization of Jerusalem artichoke tuber puree showed that fructose and inulin hydrolysed by HCl 2N represented 20.6 % and 39.8 % of total solid, respectively. Meanwhile, 6.8 %, 7.9 % and 3.5 % of total solid in tubers were in forms of hemicelluloses, cellulose and lignin. About 12.7 % of inulin has not been hydrolysed by HCl and assumed to be trapped in other carbohydrate polymers (*e.g.* hemicelluloses, cellulose) structures. The bio-accessibility of these different parts of carbohydrate in raw materials of Jerusalem artichoke tubers has been previously studied in BHP batch operation, but no evidence has been revealed so far in continuous operation fermentation process.

In addition, the Jerusalem artichoke tuber is a very attractive biohydrogen production feedstock. They indeed have shown relatively high biohydrogen potentials according to the previous results obtained on maximum hydrogen potentials tests (BHP) *i.e.* 120 ± 8 $mL_{H2}.g_{TS}^{-1}$ and 1.2 ± 0.1 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$. Moreover, the most promising advantages about Jerusalem artichoke plants are their high crop yields and their capacity to grow on marginal lands or in difficult climate with poor fertilization requirements (Kosaric *et al.*, 1984; Gibbons, 1989; Baldini *et al.*, 2004). The main form of carbohydrate storage found in the whole Jerusalem artichoke plants is a polymer of fructose branched in β2-1, so-called inulin. The inulin sugars represent 75 % to 91 % of sugar content in dry tubers (Matias *et al.*, 2011). The structure of di- and tri-saccharides has been proven to significantly affect hydrogen yield in batch tests and longer chains of oligomers could lead to lower hydrogen yields (Quéméneur *et al.*, 2011). So far, only few publications reported anaerobic fermentation of Jerusalem artichoke plants, and only methanogenesis was investigated (Bagi

et al., 2007; Lehtomaki *et al*., 2008). A first study was carried out in batch assays with the objective to screen out methane potentials from energy crops including Jerusalem artichoke, which showed 340 $mL_{CH4}.g_{TS}^{-1}$ (Lehtomaki *et al*., 2008). A second study reported also the possibility to degrade this plant under anaerobic conditions, with more than 100 $mL_{CH4}.g_{TS}^{-1}$ produced (Bagi *et al*., 2007). A research project has been recently proposed on the co-digestion of Jerusalem artichoke stalk and municipal solid waste (Pignatelli *et al*., 2010). In this study, only a feasibility assessment was published to describe the Jerusalem artichoke plant cultivation and the possibility of co-digestion with the organic fraction of municipal solid waste (Pignatelli *et al*., 2010). However, up to date, no experiment was reported using Jerusalem artichoke as sole substrate for biohydrogen production either in batch or in continuous fermentation process.

From an economic point of view, continuous operation can provide sustainable productivity and feasibility at industry-scale (Hussy *et al*., 2003). Among the parameters influencing the continuous fermentation processes, organic loading rate is an important one in order to optimize hydrogen production, as suggested previously in this chapter the importance of organic load related in batch tests. Indeed, the organic loading rate, *i.e*. the inlet substrate concentration divided by the hydraulic retention time, has been largely investigated with pure sugars substrates (*e.g.* glucose, sucrose) and has a substantial effect on hydrogen production and on microbial communities in sCSTR (Kyazze *et al*., 2006; Kim *et al*., 2006; Luo *et al*., 2008; Hafez *et al*., 2010; Mariakakis *et al*., 2011). A high OLR is usually attractive from an economic point of view, since it would require a lower volume of reactor. However, in practice, too high organic loading might cause substrate or end-products inhibition (Hussy *et al*., 2003). In the literature, controversial results have been reported about the relationships existing between OLR and hydrogen yields. In some studies, high OLR (6 $g_{COD}.L^{-1}.d^{-1}$ to 240 $g_{COD}.L^{-1}.d^{-1}$) impacted negatively the overall hydrogen yields (Van Ginkel and Logan, 2005b). In contrast, in other cases, high OLR (27 $g_{COD}.L^{-1}.d^{-1}$ to 80 $g_{COD}.L^{-1}.d^{-1}$) increased significantly the hydrogen yields (Zhang *et al*., 2004).

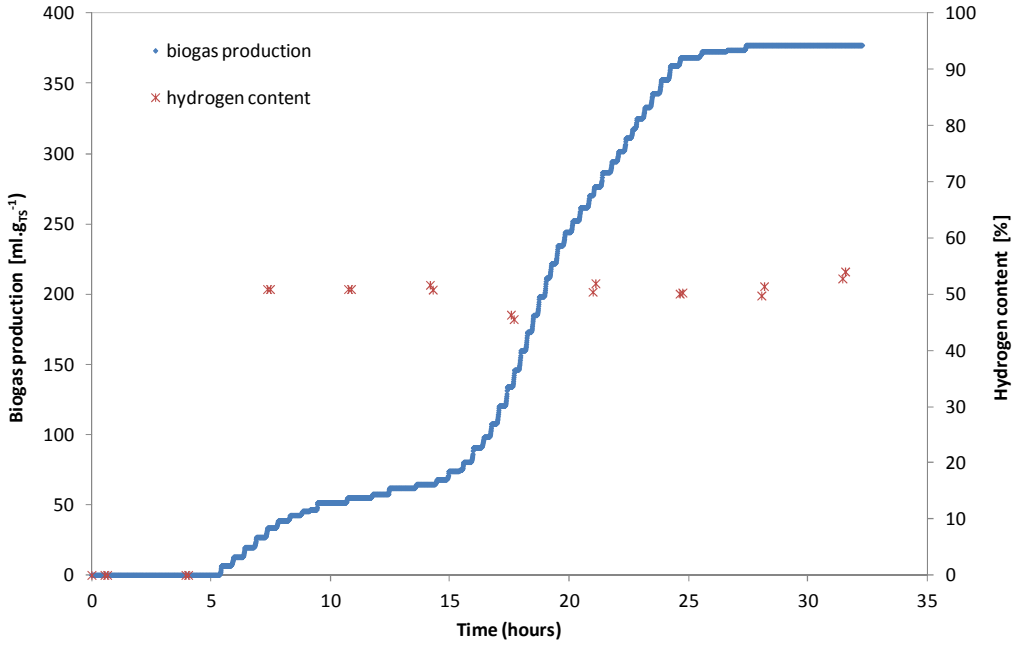
Thus, in this study, it was attempted to investigate the effect of organic loading rate on biohydrogen production and yield in CSTR using Jerusalem artichoke tubers as substrates. The dynamics of microbial community structures was also studied at different organic loading rates to better understand the relationships existing between organic loading rate, hydrogen yields and the metabolic pathways involved.

In this section, the results obtained during the start-up of the reactor in batch mode are first presented. Then, data about the hydrogen production rates, hydrogen contents in biogas and hydrogen yields were evaluated in two identical CSTR and at two different OLRs of 22 g $_{carbohydrate\ HCl}.L^{-1}.d^{-1}$ and 45 g $_{carbohydrate\ HCl}.L^{-1}.d^{-1}$.The dynamics in metabolic patterns and microbial community structures were also determined for the two OLRs.

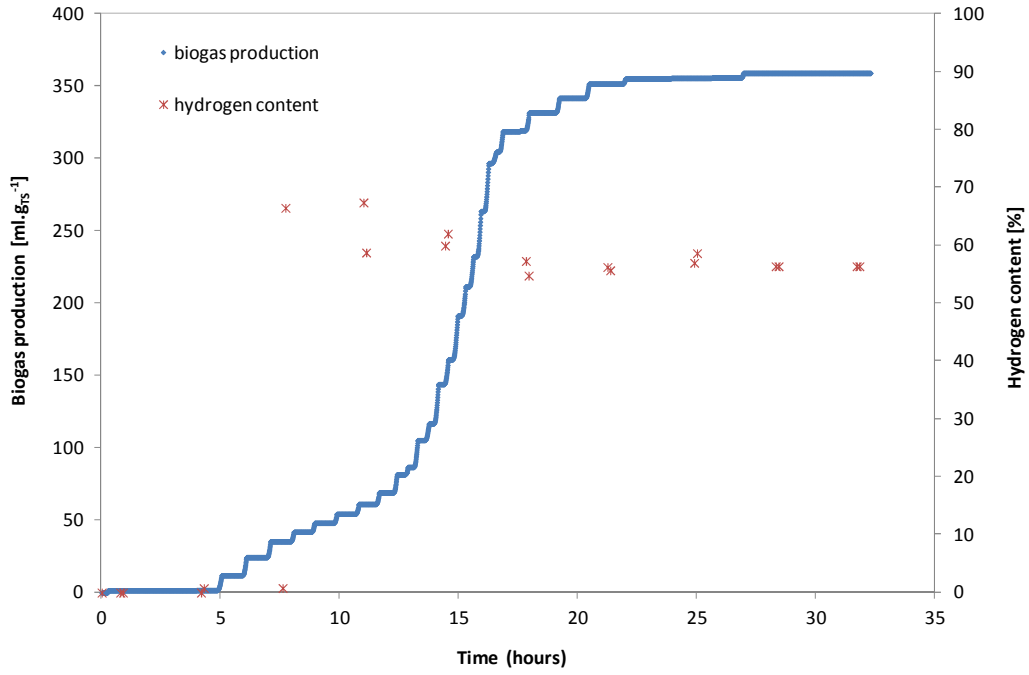
III.2.1 **Start up in batch of the CSTR**

The experiments carried out in CSTR used indigenous microorganisms from Jerusalem artichoke tubers as source of the inoculum to avoid methanogenesis that could come from the introduction of methanogenic archae from external inoculum, such as anaerobic sludge. At the start of the CSTR, a batch essay was carried out to enrich it in hydrogen-producing microorganisms and to adapt the microbial community to the tubers within the reactor and to confirm the previous biohydrogen potential measured in batch test (the maximum hydrogen accumulation per unit of substrate mass).

The start-up period of the reactor was carried out in batch mode with pH control at 5.5 and a temperature of 37 ºC. Two identical reactors with 1.5L of working volume were used and the substrate was Jerusalem artichoke tuber at 10 $g_{TS}.L^{-1}$. The cumulative biogas production and hydrogen content observed in these two batch reactors are presented in Figure III-21. Biogas of both reactors was composed of only hydrogen and carbon dioxide, with no methane, confirming our choice of inoculum.



A. variation of output biogas production and hydrogen content in reactor A.



B. variation of output biogas production and hydrogen content in reactor B.

Figure III-21. Variation of output biogas (mL.g_{TS}^{-1}) and hydrogen content (%) from the two stirred-tank reactors in batch mode feeding with 10 $g_{TS}.L^{-1}$ of Jerusalem artichoke tubers.

In reactor A, the hydrogen content in biogas reached up to 51 % after 7 hours of incubation and was constant within a range of 45 % - 51 %. Biogas accumulation was of 377 $mL.g_{TS}^{-1}$ at the end of fermentation which corresponded to a hydrogen yield of 192 $mL_{H2}.g_{TS}^{-1}$, equivalent to 1.71 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ (Figure III-21A). In reactor B, the hydrogen content in biogas rapidly increased up to 76 % after only 4.3 h of incubation and was then stable around 56 % during the exponential hydrogen accumulation phase. Biogas accumulation was 359 $mL.g_{TS}^{-1}$ in reactor B (Figure III-21B). The calculated hydrogen yield from reactor B was 201 $mL_{H2}.g_{TS}^{-1}$ and 1.79 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$.

The maximum hydrogen yields measured in the previous study (with no pH control) was 1.2 ± 0.1 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ from Jerusalem artichoke tubers (see section III-1.2.2). By comparing the hydrogen yields with and without pH control, a strict pH control led to higher yields of biohydrogen (ANOVA, *p*-value=0.02). Indeed, if pH was not controlled, initial acid pH allowed a rapid hydrogen production but also a rapid pH depletion caused by VFAs accumulation which began to inhibit microorganism growths and hydrogen production (Khanal *et al*., 2004). Higher hydrogen yields have also been observed in a previous study with pH control in batch and showed that pH of 5.2 and 5.5 were observed to be optimal pHs for hydrogen production from glucose and starch (Masset *et al*., 2010). Our results are consistent with these observations showing that keeping a constant pH can enhance the hydrogen yields.

Table III-5. Metabolic product patterns of the two batch reactors carried out with Jerusalem artichoke tubers as substrate and operated at 37 ºC and under constant pH of 5.5.

	Residual hexose ($g.L^{-1}$)	Acetate (mM)	Butyrate (mM)	Propionate (mM)	Lactate (mM)	Ethanol (mM)	Theoretical Hydrogen (mM)	Measured Hydrogen (mM)	Measured hydrogen/ Theoretical hydrogen (%)	Estimated Acetate produced by homoacetogens/T otal acetate (%)
Reactor A	n.d.	18.30	25.99	n.d.	3.97	7.40	88.59	66.24	75	20
Reactor B	n.d.	21.03	30.17	n.d.	4.45	8.71	102.39	87.22	85	12

n.d.: no detected

The associated patterns of metabolic end-products determined at the end of the batch test are presented in Table III-5. The metabolic products patterns were similar in the two reactors. The main metabolic products were acetate and butyrate, 80 % of the total soluble metabolic products (in mmol), corresponding both to hydrogen-producing pathways. Concomitantly, small amounts of lactate and ethanol were produced. In the previous subchapter BHP tests, butyrate and acetate represented 86 % of metabolic products and only lactate appeared at the end of BHP bottles from Jerusalem artichoke tubers, which is consistent with the present results.

The theoretical hydrogen accumulation by H_2-producing bacteria based on butyrate and acetate concentrations were 88.59 mM and 102.39 mM in the two reactors. This value was calculated according to equation (III-5). Equation (III-5) is based on the stoichiometric production of hydrogen from the acetate and butyrate pathways, *i.e.* 2 mol of hydrogen per mole of acetate or butyrate produced. The acetate production by homoacetogens was calculated according to Equation (III-6) proposed by Arooj *et al* (2008) to assess acetate production from homoacetogenic bacteria. The acetate produced by homoacetogenic bacteria represented 20 % and 12 % of total acetate concentration in reactor A and B, respectively.

$$H_{2\ theoretical} = 2^* [Butyrate] + 2^* [Acetate] \quad \text{(III-5)}$$

$$[Acetate_{homoacetogens}] = (2^*[Acetate] + 2^*[Butyrate] - [Propionate] - H_2)/6 \quad \text{(III-6)}$$

where [Butyrate] and [Acetate] are the concentrations measured in mM, H_2 (in mM) was the measured accumulation of H_2 expressed under experimental conditions (0 ºC, 1atm).

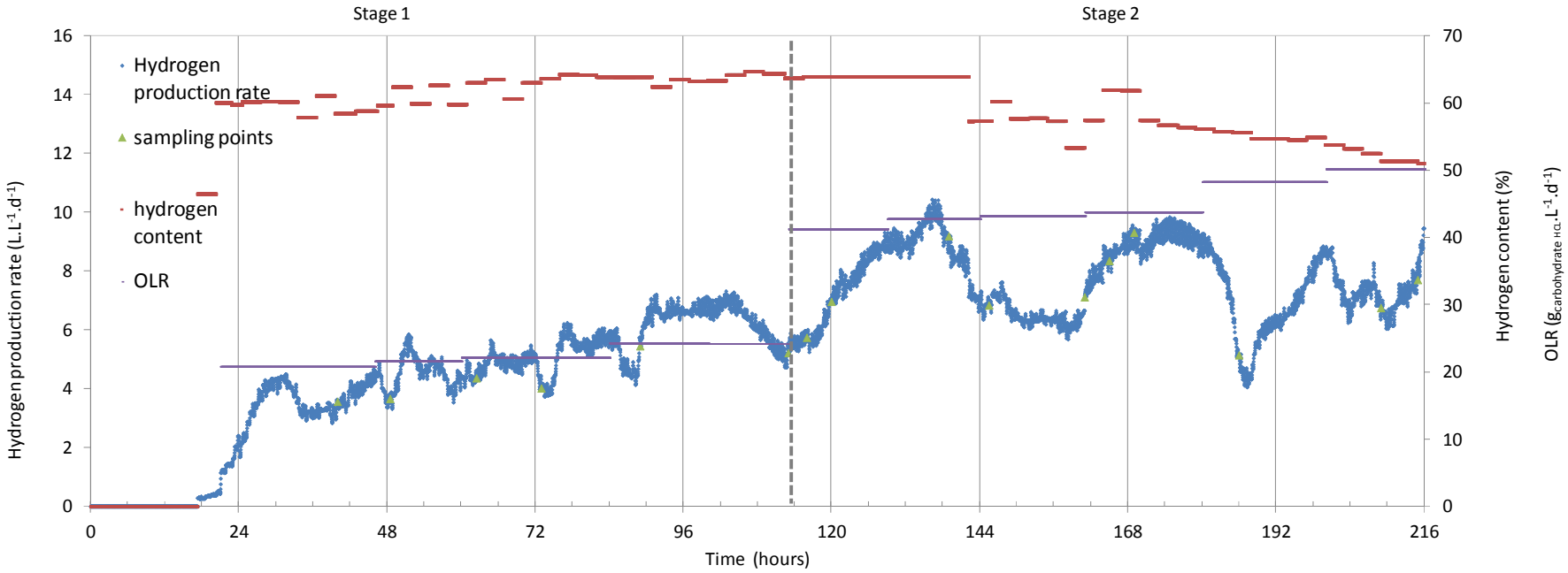
The general disadvantage of mixed culture technique is the existence of hydrogen-consuming or dissimilative microorganisms such as methanogens, homoacetogens, and lactic acid bacteria resulting in lowering the hydrogen yields in comparison with pure cultures (Hussy *et al*., 2003; Nath and Das, 2004). In our case, using the indigenous microorganisms from the Jerusalem artichoke was likely favourable to avoid the presence of methanogens, but homoacetogens were still present in this mixed culture and consumed partly the hydrogen. The lower proportions of homoacetogenic bacteria producing acetate might explain the higher hydrogen contents and hydrogen yields in reactor B.

In the precedent section, PLS regression analysis led to a simple linear equation (III-4, page134) and a multi-linear equations (III-3, page 132) to predict hydrogen yield ($mL_{H2}.g_{TS}^{-1}$) respectively from only easily soluble carbohydrates content ($Carbo_{HCl}$ in $g.g_{TS}^{-1}$) and from

easily soluble carbohydrates and protein content and the concentration of metabolites produced (*e.g.* acetate, butyrate, lactate). Applying the two prediction equations on this start-up in batch test, the predicted hydrogen yield calculated with the simple linear equation (III-4) is 125 $mL_{H2}.g_{TS}^{-1}$, while if the metabolic end-products are taken into account, the predicted hydrogen yields are 147 ($mL_{H2}.g_{TS}^{-1}$) and 160 ($mL_{H2}.g_{TS}^{-1}$) for reactors A and B, respectively. The higher prediction errors of simple linear predict equation (30 % and 33 % for the two reactors) than those of multi-linear predict equation (18 % and 16 %, respectively) indicated that it was important to consider the metabolic products into the model to improve the prediction accuracy, especially when the batch tests were not been performed in the same experimental conditions.

III.2.2 **Hydrogen production at different organic loading rates**

In order to investigate the effect of organic loading rate to hydrogen yield in continuous fermentation process, two identical continuously stirred tank reactors (CSTR A and B) were operated with two feeding concentrations (10 $g_{TS}.L^{-1}$ and 20 $g_{TS}.L^{-1}$) with a constant HRT of 6 h. The two reactors were both started in batch mode with 1.5 L of Jerusalem artichoke tubers (10 $g_{TS}.L^{-1}$) and indigenous microorganisms as inoculum. Figure III-22 and Figure III-23 illustrate the hydrogen production rates and hydrogen contents in the biogas produced in the two reactors. After 20 h of incubation, the reactors were fed with a solution of Jerusalem artichoke tubers at 10 $g_{TS}.L^{-1}$ corresponding to an organic loading rate of 22 ± 1 $g_{\text{carbohydrate HCl}}$ $L^{-1}.d^{-1}$ and under a hydraulic retention time of 6 h. At this OLR, continuous operation mode lasted for 15 HRT (from 20 h to 113 h) (**stage 1**). The feeding solution was subsequently increased to 20 $g_{TS}.L^{-1}$, *i.e.* OLR 45 ± 3 $g_{\text{carbohydrate}_{HCl}}.L^{-1}.d^{-1}$ under the same HRT of 6 h and was operated for 20 HRT (from 113 h to 234 h) (**stage 2**). Both reactors were successfully operated to continuously produce hydrogen. No methane was detected all along the fermentation process.


Stage 1
Stage 2
Hydrogen production rate
sampling points
hydrogen content
OLR
Hydrogen production rate (L.L-1.d-1)
Hydrogen content (%)
OLR (gcarbohydrate HCL.L-1.d-1)
Time (hours)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70
0
24
48
72
96
120
144
168
192
216

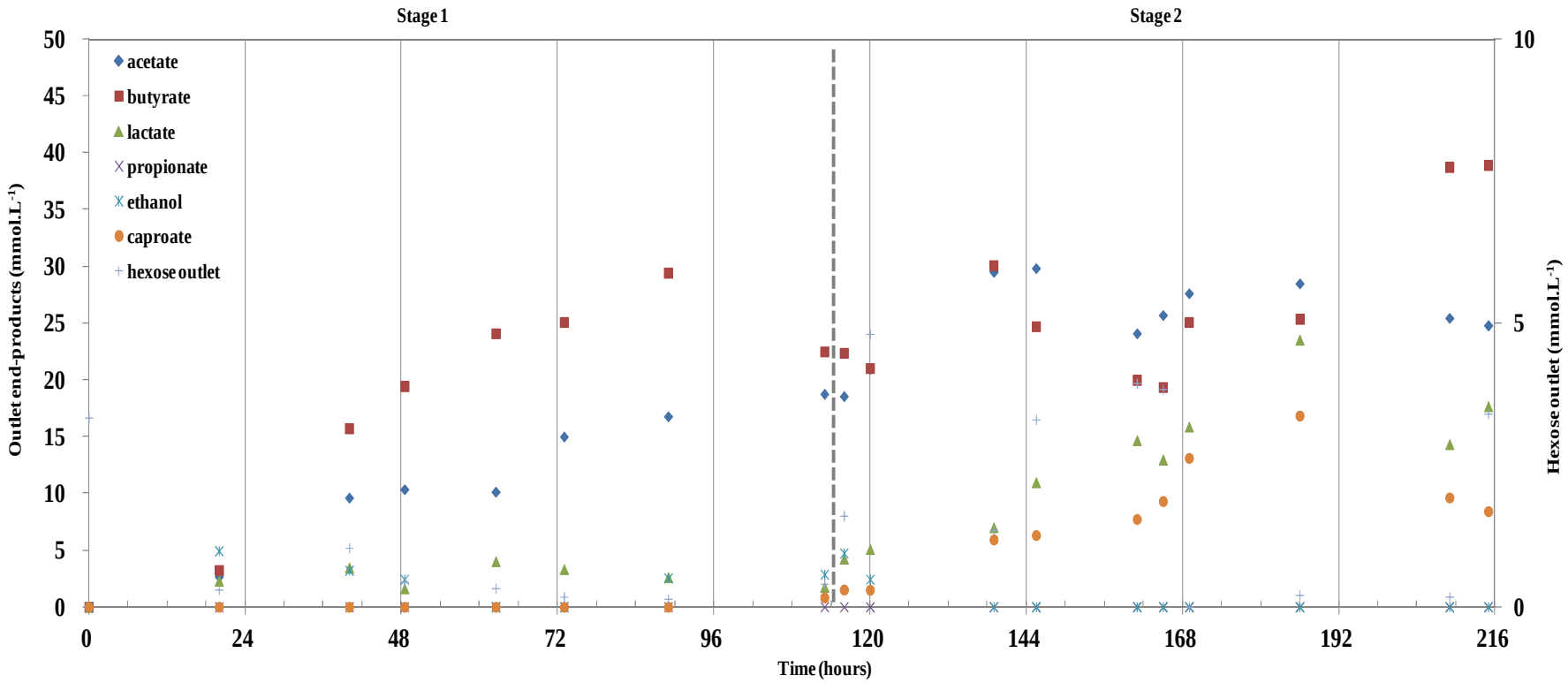
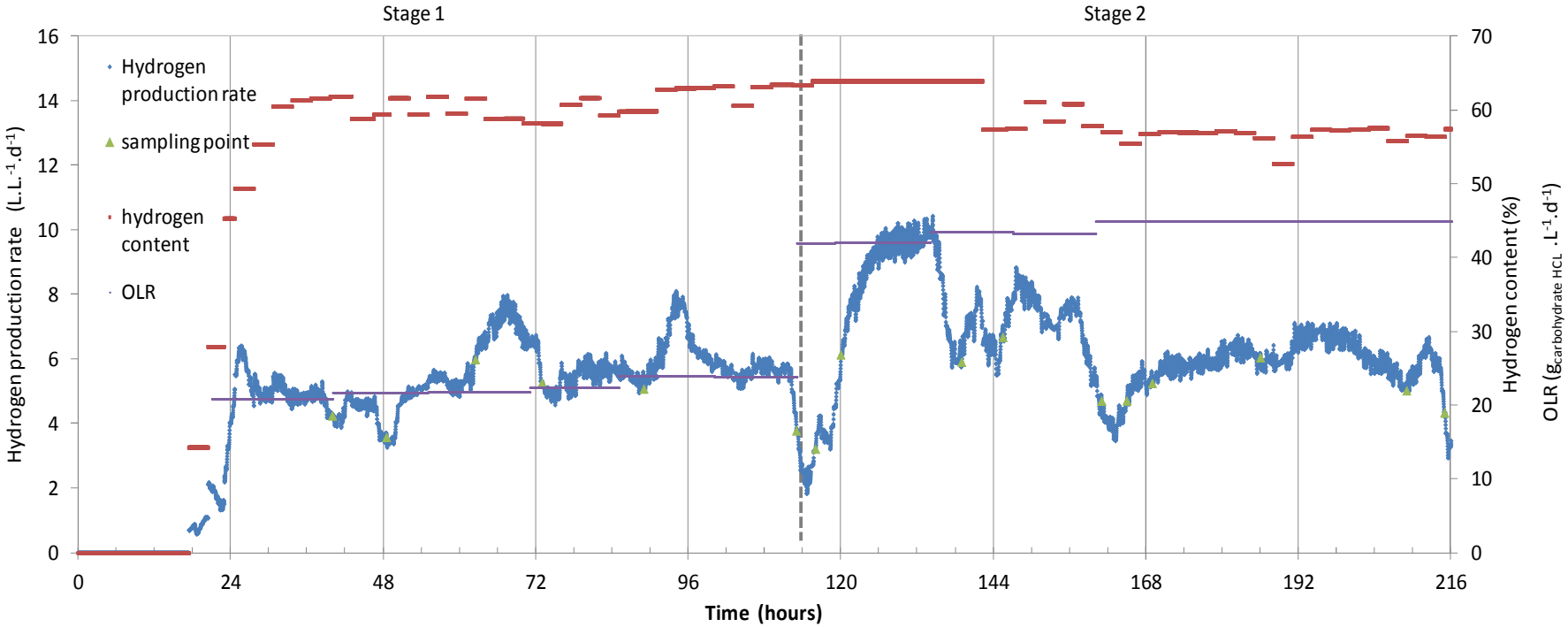


Figure III-22. The hydrogen production rate, hydrogen content and metabolic product patterns in CSTR A for two OLR


Stage 1
Stage 2
Hydrogen production rate
sampling point
hydrogen content
OLR
Hydrogen production rate (L.L.-1.d-1)
Hydrogen content (%)
OLR (gcarbohydrate HCL .L-1.d-1)
Time (hours)
0
24
48
72
96
120
144
168
192
216
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70

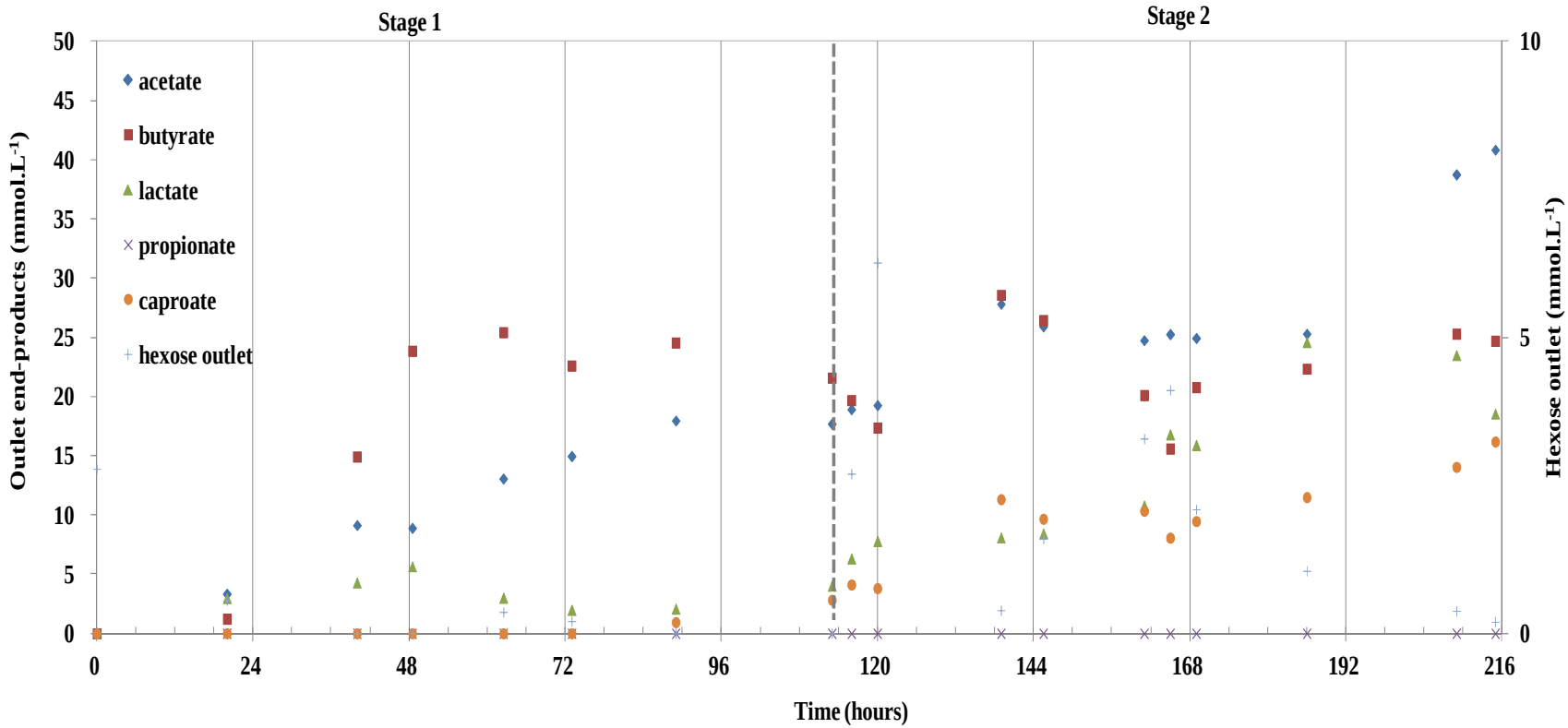


Figure III-23. The hydrogen production rate, hydrogen content and metabolic product patterns in CSTR B for two OLR.

III.2.2.1 **Results from stage 1 -reactor A**

In reactor A, the hydrogen content in biogas reached 46 % at the end of the batch mode and when the feeding started, it increased sharply to 60 % and stabilized around 63 ± 4 %. The average hydrogen production rate was 4 ± 0.6 $L.L^{-1}.d^{-1}$ between 36 h to 72 h, 6 ± 0.8 $L.L^{-1}.d^{-1}$ between 72 h and 113 h, and 5 ± 1 $L.L^{-1}.d^{-1}$ from 36 to 113 h. In this stage 1, the average VFAs concentrations reached 20 ± 4 $mmol.L^{-1}$ of butyrate and 10 ± 0.4 $mmol.L^{-1}$ of acetate during the 36 h-72 h period. Then butyrate increased to 26 ±4 $mmol.L^{-1}$ and acetate to 17 ± 2 $mmol.L^{-1}$ during the 72 h-113 h period. The slight increased of hydrogen production rate after 72 h was related to higher butyrate production. Other metabolic products such as lactate or ethanol remained at a low level for the whole stage I (<5 $mmol.L^{-1}$) (Figure III-22). Later microbial community study showed new species become co-predominant after 72 h and this new species may have enhanced hydrogen production capacity.

Notably, a punctual decrease of the hydrogen production rate at each moment of changing the storage tank of the feeding suspension was observed in both reactors likely due to a slight change in composition of the feeding solution. This observation confirmed that inlet feeding could partially be degraded even at 4 °C. The hydrogen yield of reactor A calculated from an average on the whole period OLR of 22 ± 1 $g_{carbohydrate\ HCl\ added}\ L^{-1}.d^{-1}$was 1.4 ± 0.2 $mol_{H2}.\ mol_{carbohydrate\ HCl\ added}^{-1}$.

III.2.2.2 **Results from stage 1 -reactor B**

In reactor B, the hydrogen content was only of 30 % in the biogas at the end of the start-up period in batch and gradually increased up to 60 % after 8 h of continuous operation which was consistent with reactor A. The hydrogen content was ranging from 58 % to 64 % of total biogas in outlet. Compared to reactor A, the hydrogen production rate in reactor B was stable and the average hydrogen production rate was 6 ± 0.9 $L.L^{-1}.d^{-1}$ between 36 h and 108 h. Meanwhile, the butyrate level in outlet increased gradually and kept constant during the 48 h-113 h period with an average of 24 ± 2 $mmol.L^{-1}$, while acetate concentration increased to 15 ± 4 $mmol.L^{-1}$. Other soluble metabolic products (lactate and ethanol) kept at low concentrations, < 5 $mmol.L^{-1}$ (Figure III-23). The calculated hydrogen yield in reactor B under OLR of 22 ±1 $g_{carbohydrate\ HCl\ added}\ L^{-1}.d^{-1}$ was estimated at 1.5 ± 0.3 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$.

III.2.2.3 **Results from stage 2-reactor A**

After 113 h of continuous experiment, the feeding concentration in Jerusalem artichoke tubers increased from 10 to 20 $g_{TS}.L^{-1}$ in order to double the OLR *i.e.* 45 ± 3 $g_{carbohydrate\ HCl\ added}.L^{-1}.d^{-1}$. In reactor A, the hydrogen production rate gradually increased from 5.4 to 10.1 $L.L^{-1}.d^{-1}$ during 4 HRT. After this period, the OLR increased and oscillated then regularly during the following 7 HRT between 140 h and 182 h (pseudo steady state period). The average hydrogen rate and hydrogen yield observed during this pseudo steady state period were 8 ± 1 $L.L^{-1}.d^{-1}$ and 1.3 ± 0.2 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$, respectively. During this pseudo steady state period, butyrate and acetate concentrations fluctuated around average values of 23 ± 4 $mmol.L^{-1}$ and 26 ± 3 $mmol.L^{-1}$, respectively. Simultaneously, lactate and caproate concentrations were continuously increasing from 5 up to 16 $mmol.L^{-1}$ and from 2 up to 13 $mmol.L^{-1}$, respectively. The generation of lactate and caproate were concomitant with increasing carbon dioxide content, and consequently the hydrogen content decreased from 63 % to 57 % (Figure III-22). In reactor A, the hydrogen yield (1.3 ±0.2 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$) in this stage was not significant different with the hydrogen yield (1.4 ± 0.2 $mol_{H2}.\ mol_{carbohydrate\ HCl\ added}^{-1}$) in stage 1 (ANOVA, *p*-value=0.53). .

III.2.2.4 **Results from stage 2 - reactor B**

In reactor B, there was a substantial increase of the hydrogen production rate to 10 $L.L^{-1}.d^{-1}$ once the OLR doubled and this lasted for 3 HRT. The hydrogen rate oscillated during the following 4 HRT. The average hydrogen production rate at the pseudo steady state was estimated at 7.6 ± 1.2 $L.L^{-1}.d^{-1}$. However, a pH drop from 5.5 to 4.5 occurred during 4-5 hours due to a control failure after 156 h. After this "pH-drop" event, a relative steady stage lasted for 8 HRT from 165 h to 210 h. At this stage, the hydrogen rate was 6.0 ± 0.7 $L.L^{-1}.d^{-1}$, slightly lower than that before then pH drop. Butyrate concentrations during pseudo steady states before and after the pH event were almost similar with 26.5 ± 0.1 and 23 ± 2 $mmol.L^{-1}$, respectively. In contrast, the acetate concentrations decreased from 26.9 ± 1.3 $mmol.L^{-1}$ to 16.4 ± 0.6 $mmol.L^{-1}$, respectively before and after this pH drop (Figure III-23). The hydrogen yield before and after the pH drop event were 1.0 ± 0.2 and 0.8 ± 0.1 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$. This pH drop event significantly influenced the hydrogen yields (ANOVA, *p*-value=0.01).

A sharp increase of acetate concentration during the period 209 h-216 h was observed to reach up to 40 ± 1 mmol.L^{-1} at the end of the steady period after pH drop (Figure III-23). However, no increase of hydrogen production was shown, indicating a main emergence of homoacetogens (Hussy *et al*., 2003). As a consequence, the hydrogen production rate dropped significantly, from 5.6 to 4.3 L.L^{-1}.d^{-1} and stopped in the following 2 HRT (data not shown).

III.2.2.5 **Discussion on reactor A and B performances according to the organic loading rates**

III.2.2.5.1 *Hydrogen production rates and contents in biogas*

Table III-6 summarizes the hydrogen production rates, hydrogen contents and hydrogen yields in the two reactors operated in this study. The hydrogen production rates observed in stage 1 (5.2 and 5.6 L.L^{-1}.d^{-1}) were consistent with previous results reported by Arooj *et al.* (2008) with 5.59 L.L^{-1}.d^{-1} at the same OLR (22 g.L^{-1}.d^{-1}) from starch, using a glucose-based carbohydrate equivalent to inulin. At a higher OLR of 45 g.L^{-1}.d^{-1}, the hydrogen production rates in reactors A and B were 8.0 and 7.6 L.L^{-1}.d^{-1} that is in accordance with values previously reported by Kyazze *et al*. (2006) of 7 ± 2 L.L^{-1}.d^{-1} of sucrose. Moreover, the higher variability observed in reactor A and B due to the increase of OLR was comforted by the study of Kyazze *et al*. (2006) who indicated an unsteady production of hydrogen at an OLR of 40 g.L^{-1}.d^{-1}.

In addition, the hydrogen content in biogas ranged from 57 % to 63 % (61 % in average) during stage 1 for both two reactors while, in stage 2, the percent of hydrogen decreased in both reactors with the same order of magnitude. Indeed, in reactor A, the hydrogen content decreased from 63 % to 55 % at the pseudo steady state. Similarly, in reactor B, the hydrogen content decreased from 63 % to 57 %, with average of 56 % before the pH drop and 54 % after this event. Comparable hydrogen contents in biogas were obtained by Kyazze *et al*. (2006) with 60 ± 3 % during fermentation hydrogen in CSTR from sucrose at 20 g.L^{-1}.d^{-1}. Arooj *et al*. (2008) showed similar hydrogen content (65 %) in biogas in CSTR fed with starch at 32 g_{COD}.L^{-1}.d^{-1}. Actually, when hydrogen is produced from acetate pathway, the hydrogen content should be of 67 % in biogas, while it is restricted to 50 % when the hydrogen comes from the butyrate pathway. In mixed mixed cultures, the hydrogen is produced concomitantly with both butyrate and acetate, and the hydrogen content should be

then around 55 % according to the following stoechiometric equation which is in accordance with our results (Latrille *et al*., 2011) (Eq.III-7)

$C_6H_{12}O_6 + 2H_2O \longrightarrow 3\ CH_3CH_2CH_2COOH + 2\ CH_3COOH + 8\ CO_2 + 10\ H_2$ (Eq.III-7)

Table III-6. Overview of the hydrogen production rates, H_2 content and H_2 yields obtained in two reactors after changing OLR.

	Reactor A		Reactor B		
	stage I	stage II	stage I	stage II before pH drop	stage II after
Hydrogen production rate ($L.L^{-1}.d^{-1}$)	5.2 (± 1.1)	8.0 (± 1.2)	5.6 (± 0.9)	7.6 (± 1.0)	6.0 (± 0.7)
Hydrogen content (%)	63 (± 4)	57 (± 9)	61 (± 4)	56 (± 7)	54 (± 4)
Hydrogen yield ($mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$)	1.4 (± 0.2)	1.3 (± 0.2)	1.5 (± 0.3)	1.0 (± 0.1)	0.8 (± 0.1)
Hydrogen yield ($mL_{H2}.g_{TS}^{-1}$)	109 (± 20)	116 (± 23)	102 (± 14)	78 (± 5)	64 (± 7)

In Table III-7, the hydrogen production rates and hydrogen contents found in the literature are summarized for studies carried out under similar conditions as in our study. Kim *et al*. (2006) reported the production of organic acids and alcohols at similar OLR (20 $g.L^{-1}.d^{-1}$) with less hydrogen content but with more accessible substrate of sucrose. Even lower hydrogen content (20 %) was observed in biogas in a continuous reactor operated at 10 $g_{sucrose}.L^{-1}.d^{-1}$ OLR (Mariakakis *et al*., 2011). In this study, the reactor was inoculated with un-pretreated anaerobic digester sewage and the decrease in hydrogen content was mainly due to unsteady state provoked by the presence of a recurrent methanogenic activity (Mariakakis *et al*., 2011). The authors observed also that, when OLR increased to 20 $g_{sucrose}.L^{-1}.d^{-1}$, the hydrogen content almost doubled, but, however, due to internal recirculation of the biogas for reactor mixing, it might have caused the dissolution of CO_2 to further inhibition (Mariakakis *et al*., 2011). In this context, hydrogen content in the range between 50 % and 67 % is a good indicator of a well functioning hydrogen fermentation process.

In addition, glucose concentration in outlet at 40 $g.L^{-1}.d^{-1}$ OLR was significant with 0.6 $g.L^{-1}$(*i.e.* 3.33 $mmol_{hexose}.L^{-1}$). This implied a substrate overloading or the limitation by other components, likely oligonutrients. Another explanation was proposed by Kyazze *et al*. (2006) who suggested an end-product inhibition leading to unsteady production of hydrogen for

values of butyrate and acetate higher than 163 mmol.L^{-1} and 83 mmol.L^{-1}. They suggested that these concentrations of end-products reached a lethal level, and the undissociated acid concentration of these end-products, *i.e*. 51 mmol.L^{-1} for butyrate and 27 $mmol_{hexose}.L^{-1}$ for acetate played an important inhibitory role. One explanation on the mechanism of the toxic effect of organic acids was that the high dissociated acid can lead to cell lysis by increasing the ionic strength (Van Niel *et al*., 2003), whereas the undissociated form of the acids can also penetrate the cell membrane and acts as uncouplers of proton transfer mechanisms in the cytoplasm of the cell, resulting in increasing energy requirement to maintain the intracellular pH (Jones and Woods, 1986). But the threshold value of the organic acid concentration either in dissociated or undissociated forms were not in agreement between different studies, with values of 48 to 71 mmol.L^{-1} of total butyrate proposed by Zheng and Yu (2005), a total butyrate concentration of 163 mmol.L^{-1} suggested by Kyazze *et al*. (2006) and a total undissociated acid concentration of only 10 mmol.L^{-1} reported by Van Ginkel and Logan (2005a). Based on observations in our study, if inhibition occurred, the sugar outlet concentration increased during 120 h-165 h in reactor A which corresponded to 8 ± 1 mmol.L^{-1} of total undissociated acids and 8 ± 1 mmol.L^{-1} of total undissociated acids in reactor B during 146 h-165 h, which are very low values. In conclusion, an OLR of 45 $g.L^{-1}.d^{-1}$ showed a substrate overloading resulting to sugar accumulation in outlet and significant fluctuations of the hydrogen production rates.

Table III-7. Reported values of hydrogen production rates and hydrogen contents in CSTR at different organic loading rates.

Substrate	Organic loading rate ($g.L^{-1}.d^{-1}$)	pH	Hydrogen production rate ($L.L^{-1}.d^{-1}$)	Hydrogen content (%)	References
sucrose	20	5.3	4.37 (± 0.5)	60	Kyazze *et al*.,2006
sucrose	40	5.3	6.79 (± 1.5)	56	Kyazze *et al*.,2006
sucrose	80	5.3	12.12 (± 2.7)	55	Kyazze *et al*.,2006
sucrose	100	5.3	12.60 (± 1.6)	55	Kyazze *et al*.,2006
sucrose	10	5.5	0.4	20	Mariakakis *et al*.,2011
sucrose	20	5.5	1.2	37	Mariakakis *et al*.,2011
sucrose	20	5.4	0.5	40	Kim *et al*.,2006
sucrose	30	5.4	2.2	40	Kim *et al*.,2006
sucrose	40	5.4	4	60	Kim *et al*.,2006
sucrose	60	5.4	7.8	60	Kim *et al*.,2006
starch	40	5.3	4.29	nd,	Arooj *et al*., 2008
starch	80	5.3	5.59	nd.	Arooj *et al*., 2008

III.2.2.5.2 *Hydrogen performances comparisons*

As shown in Table III-6, in stage 1, the hydrogen yields from two reactors were 1.4 ± 0.2 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ and 1.5 ± 0.3 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$, which corresponded to the average of hydrogen production rates during the pseudo steady state. No significant difference between the hydrogen yields from reactor A and reactor B was observed in stage 1 (ANOVA, p=0.47), indicating that the two reactors had similar behaviors at OLR of 22 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$. In contrast, in stage 2, the hydrogen production processes were different in the two reactors with high fluctuations. Indeed, hydrogen yields in stage 2 in reactor A during pseudo steady state was 1.3 ± 0.2 $mol_{H2}.mol_{c\ carbohydrate\ HCl\ added}^{-1}$, while hydrogen yield in reactor B in stage II was 1.0 ± 0.1 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$. A variance analysis showed

the data of hydrogen yields in reactor A and B in stage 2 were statistically significantly different (ANOVA, *p*-value=0.038). The steady state reached after the pH drop in reactor B was not considered since the operation parameters were changed. The pseudo steady state in reactor B before the pH drop was too short to conclude but suggested that higher OLR (*i.e*. 45 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$) led to lower hydrogen yield.

The best hydrogen yield of 1.5 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ observed in this study in reactor B at low OLR (22 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$) was in the range of reported hydrogen yields from other raw organic waste such as potato starch residues (Hussy *et al*., 2003), but lower than the reported values of 2.1 $mol_{H2}.mol_{glucose}^{-1}$ obtained from molasses (Fang and Liu, 2002) and 2.8 $mol_{H2}.mol_{lactose}^{-1}$ from cheese whey (Davila-Vazquez *et al*., 2008) (Table III-8). This might result from the different levels of biological accessibility to the carbohydrates in diverse substrates. Glucose is one of the easiest substrates for microorganism to convert to hydrogen, with the highest hydrogen yields (Fang and Liu, 2002), followed by sucrose (Kyazze *et al*., 2006) and starch (Hussy *et al*., 2003). Starch and inulin present similar structures and, in our study, hydrogen yields were also similar to the reported values on starch under the same operating conditions. This negative correlation between hydrogen yields and the chain length of the carbohydrate was previously shown by Quéméneur *et al*. (2011) using mono-, di-and tri-saccharides.

The average of hydrogen yield in stage 1 from two reactors was 113 ± 5 $mL_{H2}.g_{TS}^{-1}$ (*i.e*. 1.3 ± 0.1 $mol_{H2}.mol^{-1}$) was very close to hydrogen production yield obtained in previous BHP test (*i.e.* 120 ± 8 $mL_{H2}.g_{TS}^{-1}$, equivalent1.2 ± 0.1 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$). Actually, BHP test predicted well the hydrogen performance in CSTR when the organic load was in the same range (10 $g_{TS}.L^{-1}$ of Jerusalem artichoke tubers in this case). In our laboratory, other BHP and CSTR experiments in terms of hydrogen production from municipal waste confirmed this conclusion (data not shown).

Table III-8. Hydrogen yields from optimal OLR reported in the literature.

Substrate	OLR ($g.L^{-1}.d^{-1}$)		Maxi.H_2 yield	References
	investigated	optimal		
glucose	9-75	75	1.7 $mol_{H2}.mol_{glucose}^{-1}$	Lin and Chang, 1999
glucose	-	28.08	2.1 $mol_{H2}.mol_{glucose}^{-1}$	Fang and Liu, 2002
glucose	6-96	6	2.8 $mol_{H2}.mol_{glucose}^{-1}$	Van Ginkel *et al*. 2005b
sucrose	20-100	80	1.15 $mol_{H2}.mol_{hexose\ added}^{-1}$	Kyazze *et al*., 2006
starch	13-20	13.33	1.25 $mol_{H2}.mol_{hexose\ added}^{-1}$	Hussy *et al*., 2003
cheese whey	92.4-184.4	138.6	2.8 $mol_{H2}.mol_{lactose}^{-1}$	Davila-Vazquez *et al*., 2008
Inulin	22-45	22	1.5 $mol_{H2}.mol_{carbohydrate\ HCL\ added}^{-1}$	This study

III.2.2.6 **Relationship between hydrogen yields and metabolic pathways at different organic loading rates**

Table III-9 summarizes the COD mass balance in the two reactors. This COD balance was calculated on the based of the inlet organic load of 0.62 $g_{carbohydrate\ HCl}.g_{TS}^{-1}$. The recovery of COD was in range from 78 % to 101 % showing that the part of carbohydrates hydrolysable by HCl had been used to produce hydrogen in CSTR. This is consistent with the conclusion drawn from previous BHP test. The bio-accessibility of solid waste was thus the same either in batch or in continuous operation.

As shown in Table III-9, during the whole continuous operation, acetate and butyrate were the principal metabolic products (34-89 %) in both reactors. However, the ratio of butyrate and acetate concentrations decreased when OLR increased. Low hydrogen yields seemed to be associated with lactate and caproate accumulation, indicating a metabolic derivation of the H_2-potential. Since 139 h in reactor A and 89 h in reactor B, caproate started to accumulate and was continuously observed during all the following process. Caproate represented 3 %-33 % in reactor A and 9 %-35 % in reactor B at high OLR (45 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$). The increase in caproate among total soluble metabolic products was concomitant with the decrease of the butyrate/acetate ratio. This was previously reported by Quéméneur *et al* (2012). In the literature, caproate was reported as a metabolite product of *Clostridium*

kluyveri (Seedorf *et al*., 2008; Ding *et al*., 2010), *Clostridium butyricum* (Butel *et al*., 1995), *Clostridium scatologenes* (Kridelbaugh *et al*., 2009). *Clostridium kluyveri* was the unique species which used ethanol and acetate as sole energy source to generate butyrate, caproate and hydrogen (Seedorf *et al*., 2008; Ding *et al*., 2010) which is not consistent with our observations. In contrast, in mixed culture, *Clostridium scatologenes* was suggested as a hydrogen consumer by DGGE analysis in a CSTR reactor operated on sucrose at $10 g_{COD}.L^{-1}$ (Kim *et al*., 2006). Another study on a UASB reactor for hydrogen production assumed that caproate was produced directly from butyrate, H_2 and CO_2 (Yu and Mu, 2006). The stoichiometric equation proposed by (Yu and Mu, 2006) was as follow (Eq.(III-8)).

$$2CH_3CH_2OH + CH_3COO^- \rightarrow CH_3(CH_2)_4COO^- + 2H_2O \quad \Delta G_o^{'} = -77.40\ kJ.mol^{-1} \text{ (III-8)}$$

In this study, it was probably *Clostridium* spp. that produced caproate by consuming hydrogen following the reaction (III-8), but until now, one cannot conclude whether caproate was produced specifically by *Clostridium scatologenes* or *Clostridium butyricum* involved in the mixed culture. The accurate processing of pyrosequencing of the DNA from samplings in this study will further provide this information.

Lactate was always detected in reactor outlets, representing 3-9 % of COD inlet in stage 1 and 4-20 % of COD inlet in stage 2 in the two reactors (Table III-9). Lactate could be produced by *Clostridium* sp. resulting from metabolic shift (Lin *et al*., 2007). When carbon source is limiting, *Clostridium butyricum* has the ability to shift from butyrate/acetate fermentation to lactate/ethanol fermentation, but this shift has been reported as irreversible (Lin *et al*., 2007). However in our case, lactate was probably produced by lactic acid bacteria which could be present in inlet feeding. Indeed, a small amount of lactate ($<5\ gCOD.L^{-1}$) was detected in the feeding culture suggesting the occurrence of lactic acid fermentation. Lactate concentration remained at a low level in stage 1 whereas increasing lactate concentration was detected in stage 2. Jo *et al* (2007) pointed out the critical role of the genus *Lactobacillus* on blocking the hydrogen production in a continuous hydrogen fermentation reactor from food waste. In their study, the hydrogen production dropped and stopped after 55 days of continuous operation. By microbial community characterization on samples from production stage and production failure, they found out that the dominant bacteria shifted from *Clostridium acetobutylicum* and *Clostridium tyrobutyricum* to *Lactobacillus sakei,*

Lactobacillus kefiri and *Lactobacillus delbrueckii* (Jo *et al*., 2007). By maintaining storage tank at 4 °C, they also showed that continuous operation was finally successful (Jo *et al*., 2007). Indeed, lactic acid bacteria can easily survive and ferment at storage temperature *i.e*. 2-4 °C (Davies and Board, 1998). In our study, lactic acid bacteria introduced into CSTR by the feeding tank, were somehow inhibited or failed in competition with *Clostridium* spp. in stage 1 at low OLR of 22 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$, whereas co-dominance with *Clostridium* spp. in stage 2 likely occurred at higher OLR of 45 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$.

Table III-9. COD balance in two CSTR by changing organic loading rate.

STAGE	Simple names[a]	Sampling time (hours)	OLR[b] ($g_{COD}.L^{-1}.d^{-1}$)	Hexose residual (%)	Acetate (%)	Butyrate (%)	Ethanol (%)	Lactate (%)	Caproate (%)	H_2 (%)	Recovery (%)	Ratio HBu/HAc[c]
1	A1	40	22.2	4	11	45	6	6	0	10	82	1.6
1	A2	49	23.1	1	11	54	4	3	0	10	84	1.9
1	A3	63	23.7	1	11	65	0	7	0	12	95	2.4
1	A4	73	23.7	1	16	68	0	5	0	11	101	1.7
1	A5	89	25.9	0	17	73	4	4	0	13	110	1.8
1	A6	113	25.8	1	19	56	4	3	3	13	98	1.2
2	A7	116	44.0	3	11	32	4	4	4	8	66	1.2
2	A8	120	44.0	8	12	31	2	4	3	10	71	1.0
2	A10	139	45.6	2	17	42	0	6	13	13	93	1.0
2	A11	146	46.1	5	17	34	0	9	14	9	89	0.8
2	A12	161	46.7	6	13	27	0	12	17	10	86	0.8
2	A13	165	46.7	6	14	27	0	11	20	11	89	0.8
2	A14	169	51.5	0	14	31	0	12	26	11	94	0.9
2	A15	186	52.6	0	14	31	0	17	33	6	101	0.9
2	A16	209	53.6	0	12	46	0	10	18	8	95	1.5
2	A17	215	53.6	5	12	46	0	13	16	9	101	1.6
1	B1	40	22.3	0	11	43	5	7	0	12	78	1.6
1	B2	49	23.2	0	10	66	3	9	0	10	97	2.7
1	B3	63	23.2	1	14	70	0	5	0	16	107	1.9
1	B4	73	23.9	1	16	61	0	3	0	14	94	1.5
1	B5	89	25.6	0	18	61	0	3	4	12	99	1.4
1	B6	113	25.5	0	18	54	2	6	11	9	101	1.2
2	B7	116	44.8	5	11	28	0	5	9	5	63	1.0
2	B8	120	45.0	11	11	25	0	7	9	9	70	0.9
2	B10	139	46.4	1	15	42	0	6	25	8	97	1.0
2	B11	146	46.2	3	14	37	0	7	21	9	91	1.0

2	B12	161	47.9	5	13	27	0	9	22	6	82	0.8
2	B13	165	47.9	7	13	21	0	13	17	6	78	0.6
2	B14	169	47.9	3	13	28	0	13	20	7	84	0.8
2	B15	186	48.0	2	14	30	0	20	25	8	97	0.9
2	B16	209	48.0	1	21	34	0	19	30	7	110	0.7
2	B17	215	48.0	0	22	33	0	15	35	6	110	0.6

a. Samples name begun with A are the samples from reactor A, while those started with B are the samples from reactor B

b. OLR en $g_{COD}.L^{-1}.d^{-1}$ calculated by $g_{\text{carbohydrate HCl}}\,L^{-1}.d^{-1}*1.067$

c. HBu: Butyrate (mmol); HAc: Acetate (mmol)

In order to analyze how the inlet COD was distributed amongst the diverse metabolic pathways with regard to the two OLRs, all metabolite products represented as COD proportion were analyzed by a PLS regression. The hydrogen yields of all the samples were expressed in $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ and used as Y-block variables, while five explicative variables (the metabolic products) were the X-block variables.

Figure III-24 plot the two first latent variables, representing 53 % (for t1) and and 24 % (for t2), of the total X-block variability. In other words, 77 % of X-block variables were represented by the first two most important variables. Figure III-24A represents the locations of samples according to axes t1 vs t2. Figure III-24B illustrates the correlation circles. In Figure III-24A, the samples mapped by the two first latent variables illustrate that the samples from low OLR are projected at the right part, which is close to butyrate and ethanol COD flux distribution. The samples taken during high OLR were found at the left part, concomitantly with caproate and lactate COD flux distribution. Acetate and ethanol however, located closely on the t2 axe were independent of hydrogen yield, lactate, caproate and butyrate variables. Furthermore, Figure III-24B shows that butyrate COD proportion was correlated with the hydrogen yield since they are located very closely to each other. This means that, if COD flux was oriented to butyrate pathway, the hydrogen yield was positively influenced. This confirms that the butyrate concentration can be a good indicator of hydrogen yield as already shown in previous BHP study (see section III 1.2.4). This conclusion is in agreement with Arooj *et al.* (2008) who used starch for hydrogen production in a CSTR and found that butyrate alone was a better indicator than the HBu/HAc In contrast, caproate and lactate were negatively correlated to the hydrogen yield. This confirms the assumption that the caproate pathway should be linked to H_2-consuming pathways in our study (see equation III-8). Caproate had never been observed in precedent BHP test (section III-1.2.3), but appeared in CSTR operation with same feeding of Jerusalem artichoke tubers due to the differente origine of inoculum. In BHP test the anaerobic digestion sludge was used as inoculum whereas in this experiment, the

indigenous microorganisms origining from soil were used. Meanwhile, the inoculum had not been heated to enrich the spore-forming bacteria. The important abundance of lactic acid bacterial in inlet feeding played a strong competition role with H_2-producing bacterial in reactors. Although the growth of lactic acid bacteria might be limited by substrate in lower OLR of 22 g $_{carbohydrate\ HCl}L^{-1}.d^{-1}$, they became co-predominant once OLR increased to 45 $_{carbohydrate\ HCl}L^{-1}.d^{-1}$.(see III-2.3). Lactate was therefore negatively related with hydrogen yield.

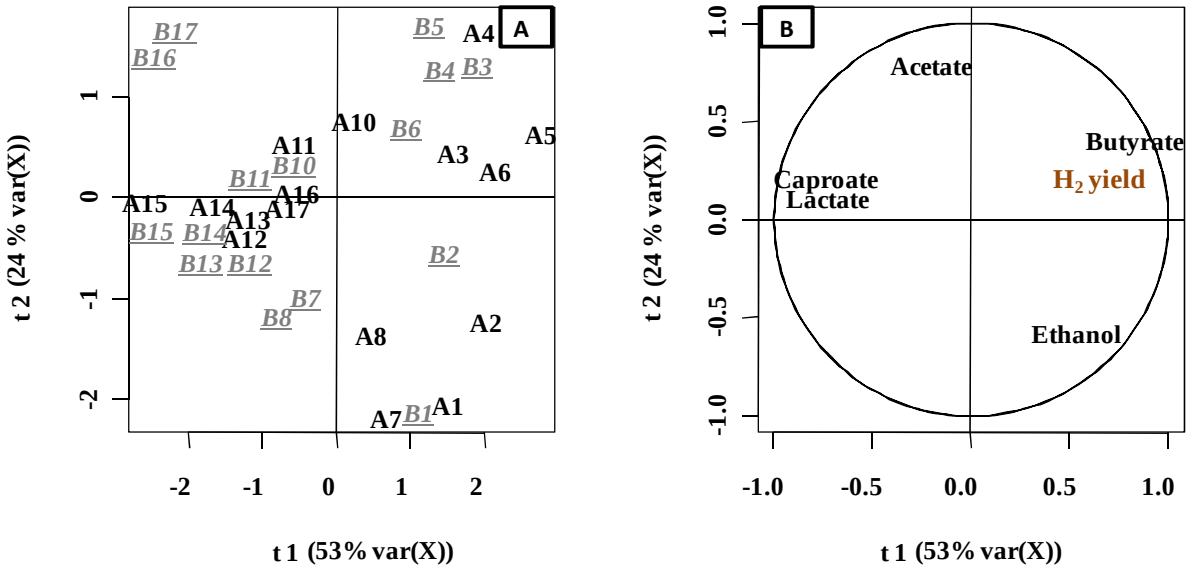


Figure III-24. Projection of 32 samplings in the two first latent factors (t1, t2) (left). The samples from reactor A were in green and the red ones from reactor B. Correlation circle of metabolic products on predicted hydrogen yield variable (right).

III.2.3 Microbial community characterization and the dynamics of the continuous biohydrogen fermentation process

Aiming to characterize the structure of the microbial community along the continuous fermentation processes, liquid samples were daily collected and then analyzed by a CE-SSCP, a molecular fingerprinting method. The profile obtained by CE-SSCP consists of a succession of peaks with area that are proportional to the abundance of one bacterial

population. These profiles are normalized and aligned with the software StatFingerprints (Michelland *et al*., 2009) in order to compare them.

The CE-SSCP fingerprinting profiles based on 16S rRNA gene diversity in samples taken at pseudo steady state are presented in Figure III-25. In this figure, the dynamics of the microbial communities are also shown for the two OLR. In stage 1, *i.e*. low OLR at 22 g $_{carbohydrate\ HCl}.L^{-1}.d^{-1}$, peak II first appeared in both reactors A and B, and then peak III emerged at the 9th HRT in reactor A and at the 7th HRT in reactor B. These two peaks were accompanied with high hydrogen production rates and yields, suggesting that they were related to *Clostridium* sp., in accordance with the position of the peaks on the left part of the profiles (data not shown - *Clostridium* sp. are commonly found in this profile zone). In stage 2, *i.e*. high OLR at 45 g $_{carbohydrate\ HCl}.L^{-1}.d^{-1}$, within the same zone of the profile, assumed to be affiliated to *Clostridium* sp., the dominant peaks changed, and finally peak I, peak II and peak III were in less relative abundance in the final mixed culture. Meanwhile, a new cluster of microorganisms (peak V) emerged (around 780 arbitrary unit of migration time). Based on previous metabolic analysis (lactate accumulation), and considering that this peak was in the right part of the CE-SSCP profile, the peak V was probably related to the genus *Lactobacillus* or *Bacillus* sp. (data not shown – Lactic acid bacteria are commonly found in this profile zone). Interestingly, this peak was also present in the inlet as the main dominant species (Figure III-26), as suggested by the low amounts of lactate found in inlet and it appeared co-dominant only at high OLR where lactate was highly accumulated. This molecular finger printing method did not allow the evaluation of the total microbial abundance due to threshold of detection. In contrast, peaks I, II, III were not detected in the inlet samples (Figure III-26), indicating that the relative abundance of these species were too low to be detected in the feeding solution. In Figure III-26, the last sample profile (pink color) showed the emergence of peak IV, which might be a homoacetogenic bacterium, since acetate concentration increased sharply with no enhancement of hydrogen production (see section III-2.2.4).

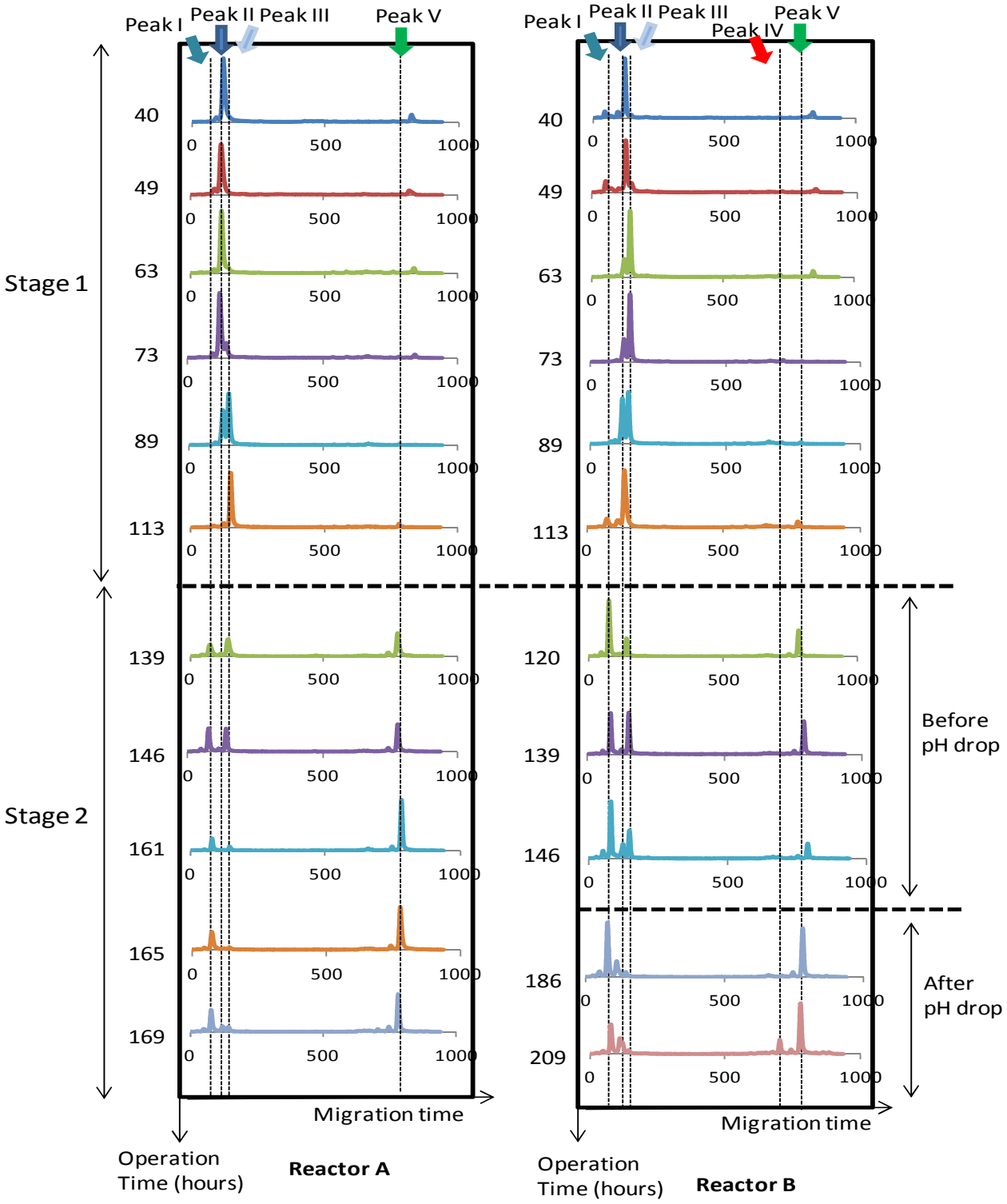


Figure III-25. Dynamics of microorganisms for two OLR in CSTR reactor A and B.

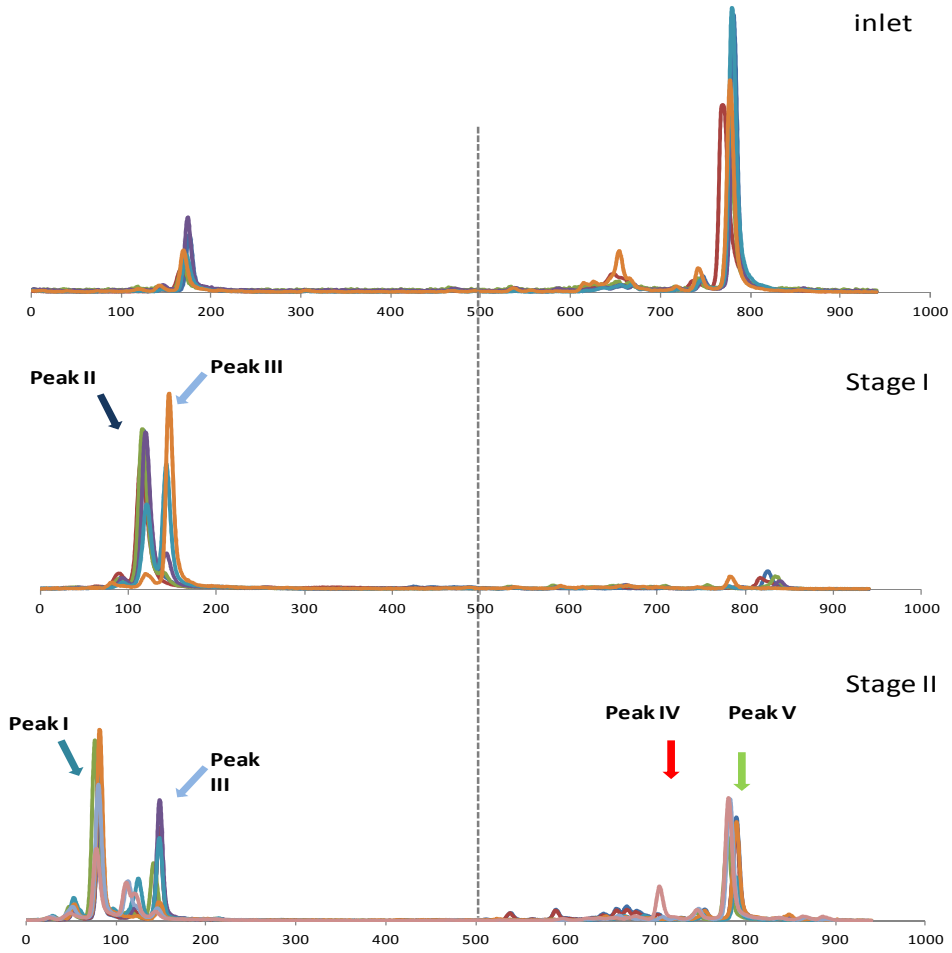


Figure III-26. Comparison of CE-SSCP profiles between the inlet and the outlet samples during OLR of 22 g $_{carbohydrate\ HCl}$.L^{-1}.d^{-1} (stage 1) and OLR of 45 g $_{carbohydrate\ \ HCl}$.L^{-1}.d^{-1} (stage 2), for both reactors .

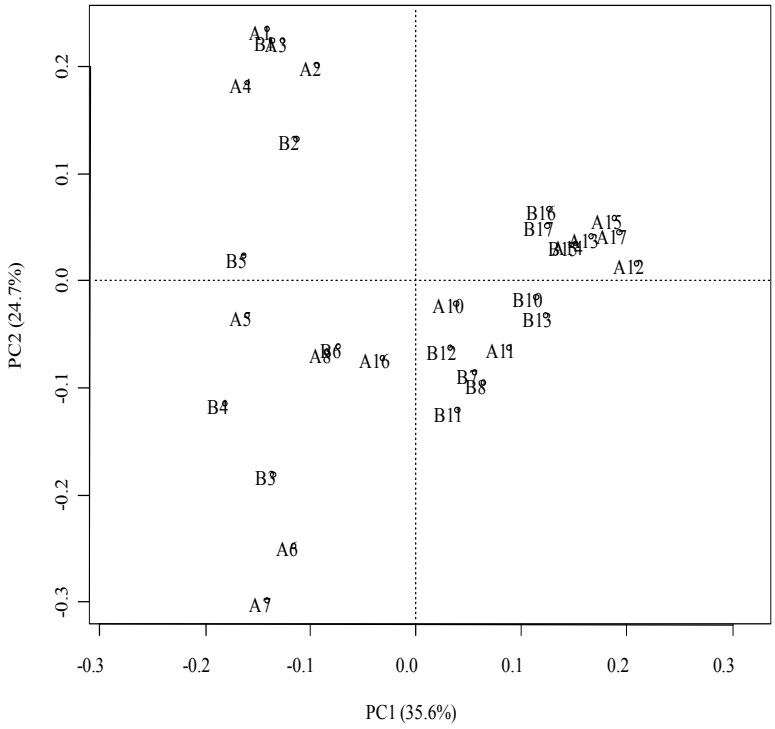
III.2.3.1 **PCA analysis of CE-SSCP profiles**

The CE-SSCP profiles correspond to a fingerprint of the overall structure of the microbial community, when comparing the appearance or disappearance of peaks, and their relative abundance over time. To estimate the difference between the profiles of CE-SSCP, an index of Euclidean distance was calculated in order to obtain a proximity matrix (Ghiglione *et al.*, 2005, Sen *et al.*, 2008). This index was calculated according to the following formulation (Legendre and Legendre, 1998).

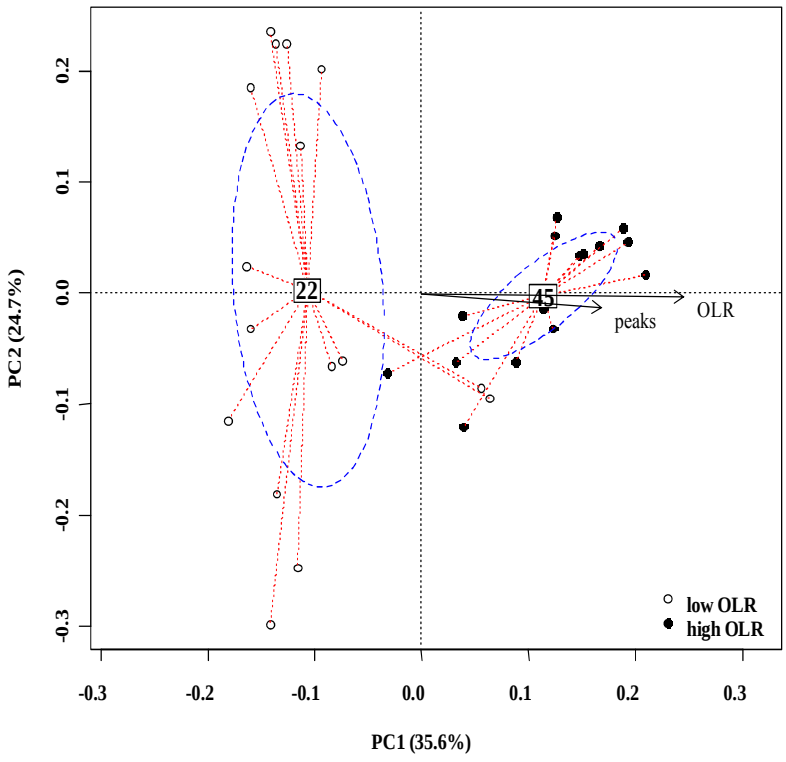
$$DE=\sqrt{\sum_{i=1}^{n}(X_k-Y_k)^2}$$

where x and y were determined by the peak positions of a CE-SSCP profile.

A principal component analysis (PCA) was used to compare the genetic distance of the species obtained in the mixed cultures according to OLR. The PCA was designed to reduce and condense the qualitative data, and then projected on a plan in order to group the cluster of data. In the PCA plot, each point represents a profile of CE-SSCP. The closest the points, the closest the genetic distance between the two corresponding samples. In addition, the factors which influence the genetic distance between the samples can be ordered by arrows which represent the correlations between one environmental factor and the CE-SSCP profiles. The length of these vectors represents the proportional gradient strength between the data of PCA and factors. Thus, longer are the arrows, higher is the correlation between the factors and the genetic distribution of the samples.



A. PCA on CE-SSCP genetic profiles.



B.Genetic profiles grouping by organic loading rate and microbial diversity

Figure III-27. PCA on CE-SSCP genetic profiles and grouped by organic loading rate and microbial diversity.

Figure III-27A presents the PCA plotting with the first two important variables which represented 60.3 % of the total variability. The distance between two points was based on the genetic distance between two CE-SSCP profiles.

Figure III-27B illustrates how the plots can be grouped according to OLR and microbial diversity factors. The OLR values (22 or 45 $g_{carbohydrate\ HCl}.L^{-1}.d^{-1}$) are located in the centre of samples' points. The surfaces of oval curves showed the total dispersion of the samples. It is here clearly demonstrated that the OLR played a selection role in the mixed culture since the samples from the two OLR were distinctly separated. Furthermore, the relationship between the structure of the bacterial communities and OLR was assessed. Indeed the number of dominant species increased with increasing of OLR (*p*-value=0.012), indicating an increase in number of peaks *i.e.* microbial diversity. The directions of the vectors representing the OLR and number of peaks factors were similar, indicating that the microbial diversity increased with OLR. This observation confirmed as well the more complex metabolic patterns found at high OLR.

Finally, Figure III-28 presents the vectors influencing the microbial genetic distributions. The angles between the arrows indicated the correlations and the length of arrows showed the significances of the vectors. As shown in this figure, the hydrogen yield was negatively correlated with OLR (*p*-value=0.004) whereas the butyrate positively correlated with the hydrogen yield with *p*-value of 0.01, and caproate and lactate were significantly inversely correlated to the hydrogen yields (*p*-value<0.001 and 0.02, respectively). These results confirmed the conclusion from PLS analysis that hydrogen yield decreased due to the metabolite derivation from acetate and butyrate pathways to caproate and lactate production. The high correlation between OLR and caproate and lactate (both

p-value<0.001) confirmed that low OLR was more appropriate for hydrogen production, while in high OLR, with likely the presence of lactic acid bacteria, lactate and caproate are concomitant with hydrogen production. The distance between the main peaks and the metabolic products showed the species responsible to the metabolic products distribution. The species represented as peak II and III produced mainly acetate, butyrate and ethanol which is consistent with *Clostridium* sp. metabolism, as previously suggested. Peak V contributed the most for caproate and lactate production, which confirmed the assumption of its affiliation to lactic acid bacteria genera. Finally, among all the main peaks, only peak III showed a significant correlation with hydrogen yields (*p*-value<0.001). Further phylogenetic identification is required to determine exactly the affiliation of these peaks.

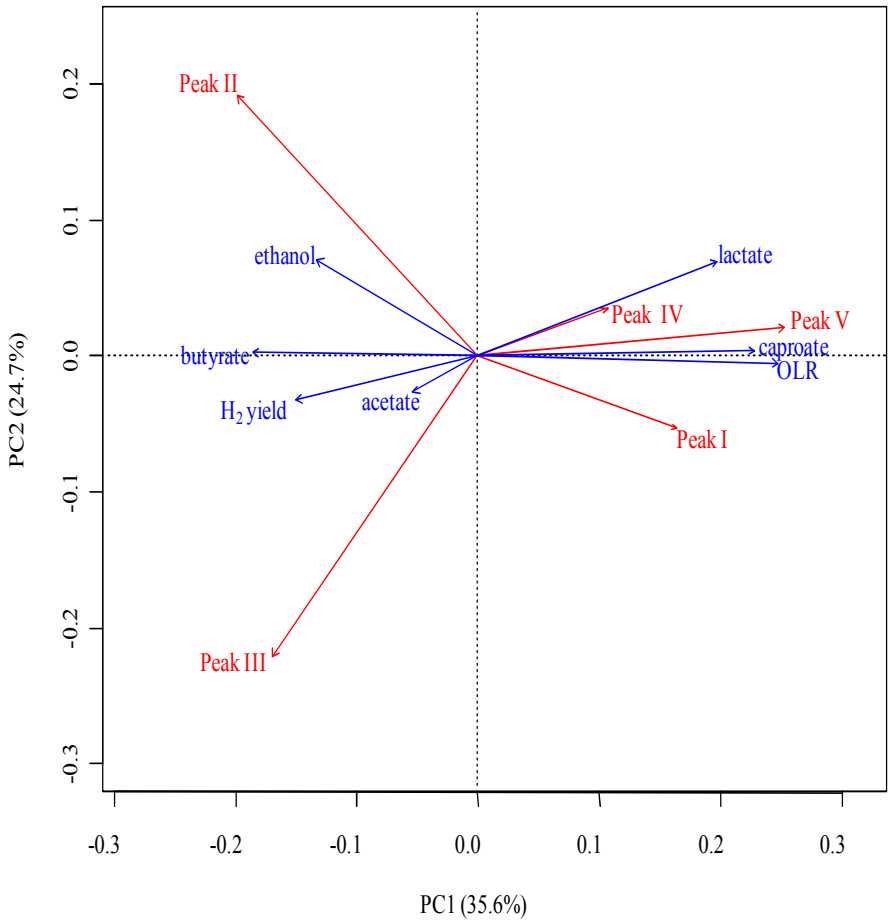


Figure III-28. The correlation of hydrogen yield and metabolic products with the change of CE-SSCP profiles.

The observation of microbial diversity increased with elevated OLR in this study was in accordance with the previous study of Hafez *et al*. (2010) who reported an increase of diversity of hydrogen producing bacteria when OLR increased from 6.5 $g_{COD}.L^{-1}.d^{-1}$ to 25.7 $g_{COD}.L^{-1}.d^{-1}$. The hydrogen producing bacteria were only *Clostridium* spp. by feeding of glucose in the range of 6.5-25.7 $g_{COD}.L^{-1}.d^{-1}$, whereas at higher OLRs of 154 and 206 $g_{COD}.L^{-1}.d^{-1}$, microbial communities shifted with coexistence of *Lactococcus* sp. and *Pseudomonas* sp. The co-predominance of lactic acid bacteria in higher OLR (*i.e.* 120g $_{COD}.L^{-1}.d^{-1}$) was also observed by Kim *et al*. (2006) using sucrose to investigate OLR effect

on hydrogen production in CSTR. The lactic acid bacteria were capable to survive even after heat treatment for spore-forming ability (Kim *et al*, 2006). Dark fermemntation should be operated at appropriate such as OLR 22 $g_{carbohydrate\ HCl} L^{-1}.d^{-1}$ to suppress the hydrogen competitors.

III.3 **Conclusion**

This chapter showed the possibility of using Jerusalem artichoke tubers in suspension to produce hydrogen with indigenous microorganisms by dark fermentation and in continuous operation. The bio-accessibility of solid substrate in reactor in terms of hydrogen production was the part of carbohydrate hydrolysable by HCl 2N, which is consistent with the observation in BHP test, showing the bio-accessibility of solid substrate was independent of reactor operation mode. A higher hydrogen yield of 1.5 ± 0.3 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ was obtained at the low OLR of 22 ± 1 $g_{carbohydrate\ HCl} .L^{-1}.d^{-1}$, in comparison with 1.0 ± 0.1 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ at OLR of 45 ± 3 $g_{carbohydrate\ HCl} .L^{-1}.d^{-1}$ in reactor B. However, in reactor A, the organic loading rate did not show significant effect on hydrogen yields (*i.e.* 1.4 ± 0.2 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$and 1.3 ± 0.2 $mol_{H2}.mol_{carbohydrate\ HCl\ added}^{-1}$ for the two OLRs).

Butyrate and acetate were the main metabolic products at low OLR, while at higher OLR lactate and caproate became co-predominant metabolic products, suggesting to dissimilative or H_2-consuming pathways were predominant at high OLR. The PLS regression showed a coherent correlation between butyrate and hydrogen yield as previously shown in BHP: among all the metabolic products, butyrate concentration correlated significantly with hydrogen yield and it was suggested to be a good indicator of hydrogen yield. The 16S rRNA gene based CE-SSCP profiles provided useful information on the microbial community structure in a continuous hydrogen fermentation process. The increased OLR had a significant effect on the microbial diversity by favoring the

implementation of other competitive microorganisms not producing hydrogen, *i.e*. lactic acid bacteria.

GENERAL CONCLUSION AND OUTLOOK

The main objective of this thesis was to study the effect of substrate composition in dark fermentation by mixed culture in term of hydrogen production, metabolite patterns and evolution of microbial communities. Fermentative hydrogen gained a great attention in recent year due to its most environmental friendly energy carrier. Meanwhile the end-products concomitant to hydrogen in dark fermentation such as VFAs are value-added chemical products. The conversion of value-less biomass, especially abundant and cheap agriculture waste, into value-added products involving hydrogen and VFAs has thus been investigated by researchers. However, the relationship between the substrate composition and hydrogen and VFAs production still remains unclear. In order to contribute to clarify this point, biohydrogen potential tests were carried out with a large range of agriculture waste in the first part of this study.

The maximum hydrogen accumulated per unit of initial added dry matter was significantly influenced by substrates categories. The carbohydrate-rich substrates were the most favorable for hydrogen production while protein-rich substrates showed the lowest hydrogen potentials. The agricultural residues and agri-industrial waste were potentially interesting substrates for hydrogen production. Hydrogen yields were correlated with the carbohydrate contents easily hydrolysed and HCl 2N hydrolysis was recommended as reference method to detect bio-available carbohydrate content for hydrogen production. Our results may be useful for waste management. Indeed, before elaborating a strategy for treating organic solid waste as well as producing hydrogen, it is therefore recommended to analyse their composition, especially to quantify easily hydrolysable carbohydrates and our prediction model may be used to estimate hydrogen potentials from the target waste.

In term of metabolic products, butyrate but not acetate was directly correlated with hydrogen yield. This conclusion was also confirmed by the results from further experiments

in CSTR. Butyrate level is therefore important to indicate a good functional hydrogen fermentation process.

Based on BHP test results, Jerusalem artichoke plant was chosen for further experiments in CSTR for its high biohydrogen potentials. Since the carbohydrate was the most important factor for hydrogen production, two concentrations of carbohydrate feeding based on same HRT (*i.e.* two OLR) were tested to investigate the dynamic of hydrogen and microbial community in CSTR. Runs on Jerusalem artichoke tubers in CSTR showed that at lower OLR, butyrate and acetate represented the main soluble metabolic products in the effluent and higher hydrogen yield was observed. This indicated that lower OLR was more appropriate for hydrogen production. Indeed at higher OLR, lactate and caproate became important soluble metabolic products beside of acetate and butyrate, indicating that the hydrogen competition pathway (*i.e.* lactate) and hydrogen consuming pathway (*i.e*. caproate) implanted at higher OLR. In consequence, hydrogen yields at higher OLR were relatively lower.

Data from microbial community dynamic analysis demonstrated that a new cluster of microorganisms emerged and became co-predominant at higher OLR related to the lower hydrogen yields. In other hand, Clostridia, composed the only cluster of microorganisms at lower OLR. The concentration of bio-available carbohydrates limited the growth of lactic acid bacteria and maintained Clostridia as dominant microorganisms. Moreover, the different species belonging to *Clostridium* genus shifted at low OLR but did not significantly influence hydrogen yields. Thus the overall microbial community analysis results suggested reduced microbial diversity towards to Clostridia could enhance the hydrogen production. Information on identifying dominant species in these microbial communities will be needed for further conclusion. Indeed, pyrosequencing of total DNA of simples targeted is currently analyzed.

The above results from this thesis provided interesting perspectives in two points: the first concerns process engineering; the second the microbial community investigation field. In this study, the linear relationship of easily hydrolyzed carbohydrate with hydrogen production was demonstrated. In terms of process engineering, the pretreatment of substrate to improve the solubilization of carbohydrates is therefore a critical step to valorize waste in term of hydrogen and VFAs by dark fermentation, especially for lignocellulosic substrates. However, the pretreatment should carefully take into account the characterization of carbohydrates. For example, the Jerusalem artichoke plant contains a large quantity of inulin, polymer of fructose, which forms HMF (5-hydroxy-2-methylfurfural), an inhibitor of hydrogen production after acid hydrolysis (Nguyen *et al*., 2009; Quemeneur *et al*., 2012). Thus, it would be worthy to investigate mild pretreatments of Jerusalem artichoke plant to improve inulin solubilisation and to avoid the production of inhibitors. For instance the use of biological enzymes naturally present in microorganism can be a good choice. Since parts of inulin are embedded within the matrix of lignocellulose, combination of fungi (capable to hydrolyse lignocellulose) hydrolysis stage and dark fermentation or addition of target species such as *Clostridium thermocellum* into mixed culture directly involving dark fermentation are proposed to test.

In CSTR experiments, about 60 % of total solids of Jerusalem artichoke tubers in form of carbohydrate were converted to hydrogen and VFAs and lactate. The remaining substrate and energy included in metabolic end-products must be used. An interesting further investigation can focuse on improved energy recovery. For example, combination of dark fermentation with photo fermentation or methane production may be interesting. Finally, once a stable two or three-stage procedure will be established, a pilot-scale experiment should be tested and meanwhile, “life-cycle assessment” should be calculated.

The population dynamics proved that at lower OLR, only hydrogen-producing bacteria (*e.g. Clostridium* spp.) were pre-dominant in the total microbial community and the process

showed better hydrogen production rate and performances. This fact suggests to regulate microbial community by operating appropriate OLR. Further research should deepen insights of the mechanism of reducing microbial diversity. For example, the operation parameters such as substrate concentration and composition and HRT could be changed while keeping the same OLR. Analysis of microbial community during these changing operational conditions by CE-SSCP based on the *hyd*A gene (encoding [Fe-Fe]-hydrogenases) and 16S rRNA, would permit to compare the function, dynamics and sensibility from clostridia to other bacteria.

Finally, considering the importance of hydrogen producing bacteria in mixed culture for hydrogen performance, they can be a bio-indicator of efficient fermentation process operation. The decrease of their level may indicate that the process might later deteriorate. Changing operation parameters such as reducing HRT or applying heating treatment can enrich these hydrogen producing bacteria. Different molecular techniques are recommended to monitor hydrogen producing bacteria such as FISH which can visualize and quantify the targeted bacteria and Real time PCR which detects the level of hydrogenase expression to indirectly monitor the abundance of hydrogen producing bacteria.

To conclude, this study has contributed to improve the understanding on composition of organic solid waste to hydrogen and microbial metabolite conversion, providing database for waste management, to design a better process for valorizing agricultural residues, to inspire a better control of process performance by involving butyrate and microbial community monitoring .

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Abstract

This study aims to investigate the effect of solid substrates composition on hydrogen production performances, metabolic pathways and microbial community changes in batch reactor and their dynamics in semi continuous reactors (sCSTR). Hydrogen is an ideal energy carrier which has gained scientific interest over the past decade. Biological H_2, so-called biohydrogen, can especially be produced by dark fermentation processes concomitantly with value-added molecules (i.e. metabolic end-products), while organic waste is treated. However, the effect of solid organic waste composition on biohydrogen production in dark fermentation has not yet been clearly elucidated.

In this study, a bibliographic review was made on hydrogen production from agricultural waste. This survey on literature showed that diverse performances were reported on hydrogen production due to the variability in substrate compositions and experimental conditions. After having optimized a protocol of biohydrogen potential test (BHP), a wide variety of organic solid substrates aiming to covering a large range of solid waste was tested to provide a comparable data analysis. The results of a PLS regression showed that only soluble carbohydrates or easily available carbohydrates correlated with hydrogen production. Furthermore, hydrogen yields correlated as well with butyrate H_2-producing pathway which is consistent with the literature knowledge. A predictive model of hydrogen yield according to carbohydrate content was proposed. Then, experiments were carried out in sCSTR with Jerusalem artichoke tubers as a case study. It was shown that low organic loading rate favored continuous hydrogen production while higher organic loading introduced hydrogen competition pathways and decreased the overall hydrogen yields. Moreover, 16S rRNA gene based CE-SSCP profiles showed that increasing OLR had a significant effect on the microbial diversity by favoring the implementation of microorganisms not producing hydrogen, i.e. lactic acid bacteria

(PhD thesis in English)

Defended on July 20th, 2012 at : UNIVERSITÉ MONTPELLIER 2 SCIENCES ET TECHNIQUES

with the financial support of : UNIVERSITÉ MONTPELLIER 2 SCIENCES ET TECHNIQUES

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