Application of pretreatments to enhance biohydrogen and/or methane from lignocellulosic residues: linking performances to compositional and structural features

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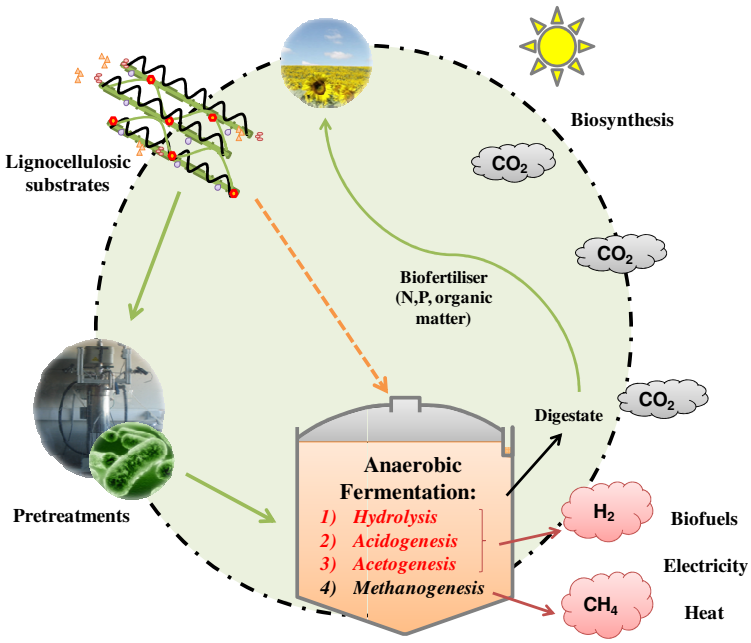

Application of pretreatments to enhance biohydrogen and/or methane from lignocellulosic residues: linking performances to compositional and structural features

Application de prétraitements pour améliorer la production d'hydrogène et/ou méthane à partir de résidus lignocellulosiques : lien entre performances et paramètres structuraux et compositionnels

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Application of pretreatments to enhance biohydrogen and/or methane from lignocellulosic residues: linking performances to compositional and structural features

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ABSTRACT

In the future, various forms of renewable energy, such as second generation biofuels from lignocellulosic residues, will be required to replace fossil fuels. Among these, biohydrogen and methane produced through fermentative processes appear as interesting candidates. However, biohydrogen and/or methane production of lignocellulosic residues is often limited by the recalcitrant structure and a pretreatment step prior to fermentative processes is often required. Up to date, informations on lignocellulosic characteristics limiting both hydrogen and methane production are limited.

Therefore, this work aims to investigate the effect of compositional and structural features of lignocellulosic residues on biohydrogen and methane performances for further developping appropriate pretreatments strategies. Firstly, a panel of twenty lignocellulosic residues was used to correlate both hydrogen and methane potentials with the compositional and structural characteristics. The results showed that hydrogen potential positively correlated with soluble carbohydrates only. Secondly, methane potential correlated negatively with lignin content and, in a lesser extent, with crystalline cellulose, but positively with the soluble carbohydrates, amorphous holocelluloses and protein contents. Pretreatments strategies were further developed to enhance both hydrogen and methane production of sunflower stalks. Dilute-acid and combined alkaline-enzymatic pretreatments, which were found efficient in solubilizing holocelluloses into soluble carbohydrates, were applied prior to biohydrogen potential tests. By combined alkaline-enzymatic pretreatment, hydrogen potential was fifteen times more than that of untreated samples. On the contrary, hydrogen production was inhibited after dilute-acid pretreatments due to the release of byproducts (furfural, 5-HMF and phenolic compounds) that led to microbial communities shift toward no hydrogen producing bacteria. Similarly, methane production, five thermo-chemical pretreatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) found efficient in delignification or solubilization of holocelluloses, were considered. Among these pretreatments, the best conditions were 55°C with 4% NaOH for 24 h and led to an increase of 29-44 % in methane potential of sunflower stalks. This pretreatment condition was validated in one stage anaerobic mesophilic continuous digester for methane production and was found efficient to enhance from 26.5% the total energy produced compared to one stage-CH_4 alone. Two-stage H_2 (batch) / CH_4 (continuous) process was also investigated. Nevertheless, in term of energy produced, no significant differences were observed between one-stage CH_4 and two-stage H_2 /CH_4.

RESUME

Dans le futur, différentes sources d'énergies renouvelables comme les energies de seconde génération produites à partir de déchets lignocellulosiques seront nécessaires pour palier à l'épuisement des énergies fossiles. Parmi ces énergies de seconde génération, le biohydrogène, le méthane et l'hythane produits à partir de procédés fermentaires anaérobies représentent des alternatives prometteuses. Cependant la production de biohydrogène et de méthane à partir de résidus lignocellulosiques est limitée par leurs structures récalcitrantes et une étape de prétraitement en amont des procédés fermentaires est souvent nécessaire. A ce jour, peu d'informations sur les paramètres limitant la conversion des matrices lignocellulosiques en hydrogène ou méthane sont disponibles.

Ce travail a pour but d'étudier l'impact des facteurs biochimiques et structurels des résidus lignocellulosiques sur les performances de production d'hydrogène et de méthane, pour pouvoir par la suite développer des stratégies de prétaitements adaptées. Tout d'abord, sur un panel de vingt substrats lignocellulosiques, les potentiels hydrogène et méthane ont été corrélés aux paramètres biochimiques et structurels. Les résultats ont mis en évidence que le potentiel hydrogène est uniquement corrélé positivement à la teneur en sucres solubles. La production de méthane quant à elle est négativement corrélée à la teneur en lignine et, à un moindre degré, à la cristallinité de la cellulose, mais positivement à la teneur en sucres solubles, holocelluloses amorphes et protéines. Par la suite, des stratégies de prétraitements ont été établies pour améliorer la production d'hydrogène et de méthane. Le couplage prétaitements alcalins/enzymatique ainsi que les prétraitements à l'acide dilué, efficaces pour solubiliser les holocelluloses en sucres solubles ont été appliqués en amont de la production d'hydrogène. En combinant le pretraitement alcalin avec une hydrolyse enzymatique, le potentiel hydrogène des tiges de tournesol fut multiplié par quinze. En revanche, suite aux prétraitements acides, la production d'hydrogène fut inhibée à cause de la libération de sous-produits (furfural, 5-HMF et composés phénoliques) engendrant un changement d'espèces bactériennes vers des espèces non productrices d'hydrogène. Pour la production de méthane, cinq prétraitements thermo-chimiques (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) efficaces pour délignifier ou solubiliser les holocelluloses ont été étudiés. Parmi ces prétraitements, la meilleure condition fut 55°C à une concentration de 4% NaOH pendant 24 h, résulant en une augmentation du potentiel méthane variant de 29 à 44 % en fonction des tiges de tournesol. Cette condition fut par la suite validée en réacteurs anaérobies continus avec une augmentation de 26.5% de la production de méthane. Un procédé à deux étages couplant la production d'hydrogène en batch suivi de la production de méthane en continu fut aussi étudié. Néanmoins, aucune différence significative en termes d'énergie produite ne fut observée entre les procédés à deux étages (H_2/CH_4) et à un étage (CH_4).

DISCIPLINE: Génie des Procédés, Biotechnologie de l'Environnement

MOTS-CLES : Voie fermentaire sombre, digestion anaérobie, prétraitements thermo-chimiques, prétraitements enzymatiques, paramètres structuraux et biochimiques.

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RESUME

Aujourd'hui, 80 % de la consommation énergétique mondiale est issue de ressources fossiles (charbon, gaz, pétrole) qui contribuent au réchauffement climatique (Saidur et al., 2011). Ces dernières sont ainsi responsables de divers dommages environnementaux : pluies acides, fonte des glaciers, augmentation du niveau d'eau des océans, perte de diversité…. De plus du fait de leur caractère non renouvelable, les énergies fossiles engendrent une inflation des prix ainsi qu'une forte dépendance vis-à-vis des pays producteurs. Suite à ces divers problèmes, l'Union Européenne a fixé comme objectif que 10 % de la consommation énergétique soit issu d'énergies renouvelables en 2020 dans le secteur des transports. Pour faire face à ces directives, diverses sources d'énergies renouvelables ont vu le jour telles que l'éolien, le solaire, la géothermie, l'hydroélectricité et les énergies issues de la biomasse. Parmi les biocarburants issus de la biomasse, on en distingue trois catégories en fonction de l'origine de la biomasse utilisée (Nigam and Singh, 2010) : (i) les biocarburants de 1ère génération sont produits à partir de la fraction alimentaire des plantes (graines, bulbes…). Les principaux biocarburants de première génération sont le bioéthanol et le biodiesel. Concernant plus spécifiquement le secteur de l'éthanol, deux pays assurent l'essentiel de la production mondiale: les Etats-Unis (57%, surtout à base de maïs) et le Brésil (32%, surtout à base de canne à sucre). L'Union Européenne est, quant à elle, davantage focalisée sur la production de biodiesel (à partir principalement d'huiles de colza et de tournesol), avec 53% de l'offre mondiale (10 milliards de litres en 2010) (Persillet, 2012). Cependant, de tels carburants présentent le désavantage d'entrer en compétition direct avec le secteur alimentaire et la culture des biomasses dédiées nécessite un fort apport en engrais (IEA, 2008 ; Chen and Khanna, 2012 ; Nigam and Singh, 2010). (ii) Les biocarburants de seconde génération sont quant à eux produits à partir de la fraction non alimentaire des plantes communément appelée fraction lignocellulosique (Nigam and Singh, 2010). Parmi les résidus lignocellulosiques, on distingue les résidus de cultures agricoles (paille, tiges, feuilles…) et les cultures énergétiques produites sur des sols non arables. A l'heure actuelle, la principale production de biocarburants de seconde génération concerne la production de bioéthanol à partir de bagasse de canne à sucre. Toutefois, divers obstacles résident dans la conversion de tels substrats souvent complexes et une étape de prétraitement est souvent requise en amont du procédé de fermentation (Taherzadeh and Karimi, 2008). (iii) Enfin, les bioénergies de troisième génération sont produites à partir de microalgues mais restent à l'heure actuelle au stade expérimental (Dragone et al., 2010). Pour un futur développement à l'échelle industrielle, différents verrous doivent être résolus à commencer par l'augmentation des rendements de culture de microalgues ainsi que leurs techniques de récolte (Dragone et al., 2010).

Les résidus lignocellulosiques qui sont composés de trois fractions majoritaires, ie la lignine, la cellulose et les hémicelluloses, représentent une ressource intéressante car les holocelluloses (cellulose et hémicelluloses) sont des fractions riches en sucres qui peuvent être utilisés pour la production de biocarburants tels que le bioéthanol, le méthane ou l'hydrogène (Taherzadeh and Karimi, 2008 ; Monlau et al., 2012). Parmi ces biocarburants, l'hydrogène et le méthane produits par voie fermentaire microbienne présentent l'avantage de pouvoir convertir à la fois la cellulose et les hémicelluloses, contrairement au bioéthanol produit seulement à partir de cellulose (Monlau et al., 2012). Le méthane est produit lors d'un procédé appelé digestion anaérobie au cours duquel la matière organique complexe est convertie en biogaz, un mélange de méthane (55-75%) et de dioxyde de carbone (25-45%) par des microorganismes en conditions anaérobies. Ce processus est composé de quatre étapes : l'hydrolyse qui transforme la matière organique complexe en matière organique soluble; l'acidogénèse où se convertit la matière organique soluble en acides gras volatils, alcools, hydrogène et dioxyde de carbone; l'acétogénèse où se convertissent les alcools et les acides gras volatils en acétate et un mélange d'hydrogène et de dioxyde de carbone; enfin l'acétate ainsi que le mélange d'hydrogène et dioxyde de carbone sont convertit en biogaz au cours de la dernière étape de méthanogénèse. L'hydrogène peut être produit lors d'un procédé appelé fermentaire « sombre » qui est une partie de la digestion anaérobie au cours de laquelle l'étape de méthanogénèse est inhibée soit par prétraitements des cultures mixtes, soit par l'utilisation de souches fermentaires pures (Guo et al., 2012). En effet, il est possible d'inhiber les archées méthanogènes des cultures mixtes par un prétraitement thermique (ie 90°C, 15 min) ou par des chocs de pH (Guo et al., 2012). Dans de telles conditions, les archées méthanogènes sont inactivés alors que les bactéries productrices d'hydrogène, ie *Clostridium* ont la capacité de sporuler. Les deux voies métaboliques produisant de l'hydrogène à partir de cellulose ou hémicelluloses sont les voies de production d'acétate et de butyrate (Latrille et al., 2011). La production d'acétate entraine un rendement stœchiométrique théorique de 4 moles d'H_2 par mole d'hexose (498 NmL H_2 g hexose^{-1}), tandis que pour la voie butyrate, le rendement molaire en hydrogène est plus faible avec 2 moles d'H_2 par mole d'hexose (249 NmL H_2 g hexose^{-1}). En théorie, il est possible d'espérer une production d'hydrogène de l'ordre de 2.5 moles d'H_2 par mole d'hexose (311 NmL H_2 g hexose^{-1}) en utilisant des cultures mixtes où se produisent un mélange de ces voies (Hawkes et al., 2007). Dans le cadre de cultures mixtes, il est possible que se réalisent des voies métaboliques compétitrices ou consommatrices de la production d'hydrogène, comme les voies produisant du lactate, de l'éthanol ou du propionate. Pour rendre le procédé économiquement rentable, ces métabolites (ie acétate, butyrate, lactate, éthanol, propionate) peuvent être ensuite convertis en méthane dans un procédé en deux étapes H_2/CH_4 (Pakarinen et al., 2009 ; Pakarinen et al., 2011).

En tant que substrat de fermentation, les résidus lignocellulosiques présentent une structure récalcitrante qui limite leur conversion en hydrogène et méthane et par conséquent une étape de prétraitement est souvent requise en amont du procédé de fermentation anaérobie (Monlau et al., 2012 ; Taherzadeh and Karimi, 2008). Généralement, les prétraitements sont classifiés en trois grandes

catégories : physiques, thermo-chimiques et biologiques (Mosier et al., 2005). Une combinaison de ces différentes catégories de prétraitements peut aussi être envisagée (Monlau et al., 2012). Pour la production de bioéthanol il a été clairement identifié qu'il est nécessaire de solubiliser les hémicelluloses et la lignine afin de favoriser l'hydrolyse enzymatique de la cellulose en glucose pour une future conversion en bioéthanol à partir de levures *Sacharomyces cerevisae*. Pour la production de méthane et d'hydrogène, divers prétraitements ont été rapportés dans la littérature sur des substrats lignocellulosiques avec plus ou moins de réussite (Monlau et al., 2012 ; Taherzadeh and Karimi, 2008). En effet il a été montré préalablement que la teneur en lignine affectait négativement la production de méthane (Triolo et al., 2011 ; Monlau et al., 2012). Buffière et al. (2006) ont quant à eux montré que c'était la somme de la teneur en lignine et cellulose qui affectait principalement la production de méthane. Toutefois, la plupart de ces corrélations prennent en général en compte seulement un ou deux paramètres structuraux et compositionnels, et les résultats sont parfois contradictoires entre eux. Ainsi, les informations sur les paramètres structuraux et compositionnels qui pourraient limiter la conversion de matrices lignocellulosiques en hydrogène et méthane sont largement manquantes pour développer des stratégies adéquates de prétraitements.

Ces travaux de thèse se sont articulés autour de la production de biohydrogène et/ou méthane par des cultures mixtes à partir de résidus agricoles de type lignocellulosique. Les objectifs ont été tout d'abord d'identifier, sur un panel de dix-huit substrats lignocellulosiques, les paramètres structuraux et compositionnels qui limitent leur conversion en hydrogène ou méthane. Divers prétraitement thermo-chimiques et /ou enzymatiques ont été par la suite utilisés comme outils pour modifier la structure lignocellulosique. A partir de ces résultats, diverses stratégies de prétraitements ont été mises en place pour améliorer les productions d'hydrogène ou de méthane. Pour finir, dans un souci de se rapprocher de la réalité industrielle, les prétraitements les plus convaincants furent appliqués en réacteur continu.

Dans un premier temps, l'impact de divers paramètres structuraux et compositionnels des matrices lignocellulosiques sur la production de biohydrogène et méthane a été étudié sur un panel de dix-huit substrats lignocellulosiques. Les paramètres structuraux et compositionnels suivants ont été déterminés pour l'ensemble des substrats lignocellulosiques : cellulose cristalline (2.5 à 16.3 % TS), holocelluloses amorphes (7.5 à 50.3 % TS), lignine (12.3 à 35 % TS), protéines (2.3 à 29.7 % TS), sucres solubles (0 à 59.1 % TS) et acides uroniques (0.2 à 7 % TS). De plus, leurs potentiels en hydrogène (1.6 à 120 mL H_2 g^{-1} TS) et en méthane (155 à 300 mL CH_4 g^{-1} TS) ont été mesurés lors d'essais en batch. Par l'utilisation de régressions multilinéaires (logiciel Unscrambler-Version 10.2, CAMO), les potentiels hydrogène et méthane ont été reliés à l'ensemble des paramètres structuraux et compositionnels. Le caractère innovant de cette première étude repose sur le fait qu'un ensemble de paramètres structuraux et compositionnels ait été pris en compte par rapport à l'utilisation d'un seul ou deux paramètres tel que présentés dans les autres études de la littérature. Ceci a permis de mettre en évidence que seule la teneur en sucres solubles affecte significativement et positivement la production d'hydrogène de substrats lignocellulosiques. Récemment,

Guo et al. (2012) ont confirmé la forte corrélation (R^2 = 0.87) existant entre le potentiel hydrogène de divers résidus solides et leur teneur en sucres solubles (extraitx avec de l'acide chlorhydrique 2N). En ce qui concerne le potentiel méthane, il a été montré que la teneur en lignine, et à un moindre degré, la teneur en cellulose cristalline, affectent significativement et négativement le potentiel méthane des résidus lignocellulosiques. En revanche, la teneur en sucres solubles et, à un moindre degré les teneurs en protéines et holocelluloses amorphes, affectent significativement et positivement le potentiel méthane des résidus lignocellulosiques.

Dans la seconde partie, divers prétraitements thermo-chimiques (NaOH, $Ca(OH)_2$, H_2O_2, $FeCl_3$ et HCl), enzymatiques (mixture de cellulases et hémicellulases) ou une combinaison des deux ont été appliqués pour modifier les paramètres structuraux et compositionnels des matrices lignocellulosiques. Les tiges de tournesol ont été sélectionnées pour étudier l'influence des prétraitements en raison de leur structure récalcitrante (faible teneur en sucres solubles, forte teneur en lignine). Pour les prétraitements thermo-chimiques, les conditions opératoires (température, temps, concentrations) ont été choisies en fonction de la littérature (Mosier et al., 2005 ; Monlau et al., 2012b, Taherzadeh and Karimi, 2008). Les prétraitements alcalins et oxydatifs ont été effectués à basse température (55°C) pendant 24h et à une concentration de 4% (w/wTS) alors que les prétraitements acides ont été réalisés à haute température (170°C) pendant 1h et à une concentration de 4% (w/wTS) pour HCl et 10% (w/wTS) pour $FeCl_3$. Les prétraitements alcalins et oxydatifs ont été efficaces pour diminuer la teneur en lignine d'environ 30 % alors que les prétraitements acides ont été plus efficaces pour solubiliser les hémicelluloses (environ 90%) et les acides uroniques. Toutefois aucun des prétraitements thermo-chimiques étudiés ne fut efficace pour diminuer la teneur en cellulose cristalline. Les prétraitements enzymatiques ont permis de solubiliser les holocelluloses avec respectivement 11 % de solubilisation des hémicelluloses et 34 % de la cellulose. Un prétraitement alcalin (55°C, 24 h, 4% NaOH w/w) a permis d'augmenter l'hydrolyse enzymatique sur la phase solide avec respectivement 45 % de solubilisation de la cellulose et 54 % des hémicelluloses. Divers facteurs comme une restructuration de la lignine, une solubilisation des acides uroniques et aussi une faible augmentation de la surface accessible et de la porosité peuvent expliquer l'augmentation de l'hydrolyse enzymatique après le prétraitement alcalin. A partir des résultats de la première partie et de celle-ci, des stratégies de prétraitements ont alors été définies pour améliorer la conversion des tiges de tournesol en hydrogène et méthane. Etant donné que les prétraitements acides, enzymatiques ou le couplage alcalin-enzymatiques furent efficaces pour solubiliser les holocelluloses, ceux-ci furent testés pour augmenter la production d'hydrogène à partir de tiges de tournesol. Pour améliorer la production de méthane, l'ensemble des prétraitements thermo-chimiques qui agissent soit sur la délignification ou la solubilisation des hémicelluloses furent étudiés.

Dans la troisième partie, des prétraitements à l'acide chlorhydrique furent d'abord testés pour améliorer la production d'hydrogène à partir de tiges de tournesol. Pour ce faire, un plan d'expérience fut mis en place pour tester diverses températures (142 à 198 °C) et concentrations en acide (0 à 4 % HCl

(w/w)). Pour toutes les conditions testées, un potentiel hydrogène nul fut observé au bout de 10 jours de fermentation sombre en batch contrairement aux 2.3 mL H_2 g^{-1} VS observés pour les tiges de tournesol sans prétraitement. Ceci a pu être expliqué par la génération de sous-produits (furfural, 5-HMF et composés phénoliques) dans l'hydrolysat des prétraitements acides qui peuvent inhiber la croissance bactérienne (Palmqvist and Hahn Hagerdal, 2000). Dans l'hypothèse que cette inhibition provenait de la formation de sous-produits issus principalement des composés de dégradation du glucose ou xylose ie furfural et 5-HMF ou des composés phénoliques issus de la dégradation de la lignine, la condition suivante (170°C, 1h, 4% HCl w/w) fut sélectionnée pour caractériser l'impact des sous-produits présents dans l'hydrolysat (phase liquide). Outre la présence de sucres solubles tels que le glucose (0.28 g L^{-1}) et le xylose (3.14 g L^{-1}), des concentrations de 0.02 g L^{-1}, 0.13 g L^{-1} et 1.15 g L^{-1} respectivement pour les composés phénoliques, 5-HMF et furfural furent observées dans l'hydrolysat. Par conséquent, l'ajout de volumes croissants d'hydrolysat (0, 3.75, 7.5, 15 et 35 % v/v) fut étudié dans des essais de fermentation sombre en batch contenant 5g glucose L^{-1}. Pour des volumes de 0 % et 3.75 %, aucune différence significative du potentiel hydrogène ne fut observée avec, respectivement, des productions de 2.04 (±0.14) et 1.89 (±0.08) mol H_2 mol^{-1} glucose consommé. L'ajout de 7.5 % d'hydrolysat a conduit à une réduction significative de la production d'hydrogène avec un potentiel de 0.44 (±0.09) mol H_2 mol^{-1} glucose consommé. L'ajout de 7.5% en hydrolysat correspondait à des concentrations de 86 mg L^{-1}, 9 mg L^{-1} et 2 mg L^{-1} respectivement de furfural, 5-HMF et composés phénoliques dans le batch de fermentation sombre. L'ajout de 15 % et 35 % d'hydrolysat a conduit à une inhibition totale de la production d'hydrogène. Les faibles concentrations en sous-produits conduisant à l'inhibition totale de la production d'hydrogène suggèraient certainement un effet de synergie de ces composés ou la présence d'autres composés inhibiteurs non quantifiés. En effet, Quéméneur et al. (2012b) n'ont pas observé d'inhibition totale de la production d'hydrogène sur des batchs à 5g glucose L^{-1} lors de l'ajout séparé de ces composés inhibiteurs à une concentration de 1g L^{-1}. Cette inhibition du potentiel hydrogène par ajout de volume croissant d'hydrolysat fut aussi accompagnée d'un changement métabolique des voies productrices d'hydrogène (ie acétate et butyrate) vers des voies concurrentielles ou consommatrices en hydrogène (ie éthanol et lactate). En effet, l'ajout de 30% d'hydrolysat a conduit à la production de 0.08 (± 0.00) et 1.98 (± 0.05) mol mol^{-1} glucose consommé respectivement de lactate et d'éthanol. Afin d'expliquer cette inhibition du potentiel hydrogène et les changements métaboliques observés, les communautés bactériennes furent étudiées dans les différents essais de fermentation sombre par l'intermédiaire des spectres CE-SSCP. Cette technique permet de séparer les populations bactériennes et conduit à des spectres où chaque pic correspond à une espèce et son aire à la proportion de l'espèce dans le milieu. Récemment, Quéméneur et al. (2012b) et Guo et al. (2012) ont montré sur un inoculum identique que les espèces bactériennes à gauche des spectres CE-SSCP correspondaient à des espèces productrices d'hydrogène de genre *Clostridium* alors que les espèces à droite correspondent à des espèces consommatrices comme les entérobactéries et les lactobacilles. La proportion des espèces clostridies et non clostridies a été déterminée en calculant l'aire des pics respectivement à gauche et à droite des spectres CE-SSCP. Les résultats montrent que pour 0 % et 7.5 % d'ajout

d'hydrolysat, 90% des espèces présentes étaient des clostridies. En augmentant le volume d'hydrolysat les proportions d'espèces non clostridies augmentaient mais des espèces de clostridies ont subsisté dans le milieu. Par conséquent, il semble que l'inhibition du potentiel hydrogène ainsi que les changements métaboliques observés ont résulté à la fois de l'émergence d'espèces concurrentielles ou consommatrices d'hydrogène mais aussi d'une inhibition des espèces clostridies présentes dans le milieu. Etant donné que les prétraitements acides ne furent pas efficaces pour améliorer la production d'hydrogène, les prétraitements enzymatiques ainsi que le couplage alcalin-enzymatique furent étudiés. Les prétraitements enzymatiques et alcalin-enzymatique ont respectivement permis de multiplier par 13 et 21 la production d'hydrogène par rapport aux tiges non prétraitées. Cette augmentation a été expliquée en grande partie par une solubilisation croissante des holocelluloses en sucres solubles (ie xylose et glucose) après les prétraitements enzymatiques et alcalin-enzymatiques. De façon intéressante, un phénomène de diauxie des sucres solubles (ie xylose et glucose) fut observé durant le procédé de fermentation sombre. En effet, le glucose fut consommé dans un premier temps, puis le xylose par la suite. De tels phénomènes de diauxie sont couramment observés lors de l'utilisation de cultures pures comme *E. coli* ou *Saccharomyces cerevisae* mais ne sont pas répertoriés lors de l'utilisation de cultures mixtes, du fait de la grande diversité bactérienne qui permet de consommer simultanément plusieurs sucres (Hanly and Henson, 2010). Ce résultat a pu être expliqué par le choc thermique (90°C ; 15 min) appliqué à la culture mixte pour bloquer l'activité méthanogène. En effet, les spectres CE-SSCP montraient une forte sélection bactérienne lors du choc thermique avec seulement deux espèces bactériennes majoritaires présentes dans l'essai de fermentation sombre, vraisemblablement du genre *Clostridium*. A titre de comparaison, Chaganti et al. (2012) ont déterminé un nombre d'espèces bactériennes différentes allant de 114 à 164 sur des boues granulaires et non granulaires non prétraités thermiquement.

En ce qui concerne la production de méthane, les différents prétraitements thermiques (55°C, 24h ou 170°C, 1 h) et thermo-chimiques (NaOH, $Ca(OH)_2$, H_2O_2, $FeCl_3$ et HCl) furent étudiés pour améliorer la conversion des tiges de tournesol en méthane. L'ensemble des prétraitements thermiques et thermo-chimiques ont permis d'augmenter la production de méthane des tiges de tournesol par rapport au potentiel méthane de 192 (±2) mL CH_4 g^{-1} VS observé pour les tiges de tournesol brutes. Les prétraitements alcalins et oxydatifs ont permis d'améliorer en moyenne de 31% le potentiel méthane, contre 25% pour les prétraitements acides. La meilleure condition fut obtenue pour le prétraitement alcalin (55°C, 24 h, 4% NaOH) avec un potentiel méthane de 259 (±6) mL CH_4 g^{-1} VS correspondant à une augmentation de 35 % comparée aux tiges de tournesol brutes. Cette condition fut par la suite appliquée à trois autres variétés de tournesol. Le prétraitement alcalin (55°C, 24h, 4% NaOH) a permis d'augmenter les potentiels méthanes des autres variétés de 29 à 44%.

Pour finir, la meilleure condition de prétraitement obtenue pour la production de méthane en batch (55°C, 24h, 4% NaOH) a été appliquée en réacteurs continus pour évaluer sa faisabilité à l'échelle industrielle. Un couplage original à deux étages H_2 (batch) / CH_4 (continu) a aussi été comparé à un

procédé simple de production de méthane. En effet, pour rendre la production d'hydrogène rentable, il est important de la coupler à un autre procédé comme la digestion anaérobie permettant de valoriser les métabolites produits (ie acétate, butyrate, lactate, éthanol…). En terme d'énergie produite, aucune différence ne fut observée entre le procédé à un étage de production de méthane et à deux étages H_2 / CH_4. Le prétraitement alcalin a quant à lui permis d'augmenter de 26.5 % la production d'énergie totale avec une production de 1910 (±30) kWh t^{-1}VS comparé à 1520 (±40) kWh t^{-1}VS pour la configuration sans prétraitement. Les performances énergétiques ont par conséquent été comparées sur le procédé de digestion anaérobie avec ou sans prétraitement alcalin. Pour ce faire, la production totale d'énergie pour les deux configurations a été convertie en chaleur et électricité par cogénération avec des rendements respectifs de 50 % et 35 %. Dans un premier temps, on s'est intéressé à savoir si le surplus de chaleur obtenu par le procédé de digestion anaérobie suite au prétraitement alcalin serait suffisant pour couvrir la chaleur nécessaire pour chauffer la biomasse jusqu'à 55°C. En effet, dans les installations de méthanisation, la chaleur produite n'est que très peu valorisée et le surplus de chaleur obtenu pourrait donc servir pour l'étape de prétraitement. Pour la charge de prétraitement appliquée dans notre étude (35g TS L^{-1}), le bilan de chaleur est déficitaire. Toutefois différentes solutions pourraient être envisagées afin d'éviter ce déficit de chaleur comme une augmentation de la charge de prétraitement en considérant que les rendements de méthane soient les mêmes. En effet, dans ce cas la chaleur nécessaire par tonne de biomasse est réduite. Scheell et al. (2003) ont montré la faisabilité de réaliser des prétraitements avec une charge allant jusqu'à 200g TS L^{-1}. Une autre possibilité pour éviter ce déficit de chaleur serait de récupérer une partie de la chaleur de l'étape de prétraitement à hauteur de 80 % (Dhar et al., 2012). Outre la production de chaleur, le procédé de cogénération produit également de l'électricité et le prétraitement alcalin permettrait d'augmenter de 26.5 % la production d'électricité.

En conclusion, les modèles définis lors de la première partie ont permis par la suite de développer des techniques adéquates de prétraitements à la fois pour la production d'hydrogène et de méthane. Les prétraitements acides malgré leur impact sur la solubilisation des hémicelluloses en sucres solubles n'ont pas permis d'augmenter le potentiel hydrogène des tiges de tournesol en raison de la génération de sous produits principalement du furfural, du 5-HMF et des composés phénoliques. Le couplage alcalin-enzymatique semble quant à lui très prometteur afin d'améliorer les rendements de production d'hydrogène à partir de résidus lignocellulosiques. Concernant la production de méthane, l'ensemble des prétraitements thermo-chimiques se sont montrés efficaces pour améliorer le potentiel méthane des tiges de tournesol. La faisabilité de tels prétraitements a aussi été validée par la suite en réacteurs continus montrant la possibilité d'un futur développement industriel.

Ces travaux ont aussi soulevé de nombreuses perspectives:

Concernant les modèles définis pour prédire le potentiel hydrogène ou méthane des résidus lignocellulosiques, il serait désormais pertinent de les améliorer en prenant compte d'autres paramètres structuraux et compositionnels tels que la surface accessible, la porosité, les groupes acétyles ainsi que les

interactions possibles entre les composés par la détermination des complexes lignine-holocelluloses. Certains de ses paramètres permettraient certainement de comprendre encore mieux les verrous de la production d'hydrogène et méthane à partir de résidus lignocellulosiques.

Concernant la production d'hydrogène, les prétraitements enzymatiques sont efficaces pour solubiliser les holocelluloses en sucres solubles et par la suite augmenter la production d'hydrogène. Toutefois, leur développement industriel est limité par le fait que de tels prétraitements doivent être réalisés en conditions stériles afin d'éviter la reconsommation des sucres solubles par les bactéries endogènes présentes naturellement sur le substrat. Une manière d'éviter l'étape de stérilisation est de travailler en condition anaérobie par réutilisation du CO_2 issu du biogaz, qui pourrait aussi servir pour réguler le pH du prétraitement enzymatique généralement autour de 5. D'autre part, pour réduire les coûts des enzymes industrielles il serait envisageable d'utiliser des prétraitements fongiques qui peuvent sécréter des enzymes de type cellulases et xylanases. Dans un context de bioraffinerie environnementale, il serait alors intéressant d'approfondir la piste du changement métabolique vers la production d'éthanol, sous-produit d'intérêt industriel, observée en présence de sous-produits de prétraitements thermo-acides. En effet des rendements intéressants de l'ordre de 1.98 mol ethanol mol^{-1} glucose consommé ont été observés et sont relativement proches des rendements de production obtenus à partir de levures comme *Saccharomyces cerevisae*. De plus, l'utilisation de cultures mixtes est moins coûteuse et présente l'avantage de pouvoir convertir à la fois les pentoses et les hexoses contrairement à l'utilisation exclusive des hexoses par des levures du genre *Saccharomyces*. Toutefois, il faudrait étendre notre étude à des charges de fermentation plus élevées. En effet, dans notre situation, le rendement de 1.98 mol ethanol mol^{-1} glucose consommé, a été obtenu à une concentration de 5 g glucose L^{-1} contrairement à des concentrations plus élevées autour de 50 g glucose L^{-1} dans le cas de la fermentation alcoolique utilisant les levures *Saccharomyces cerevisae*.

Concernant la production de méthane, il serait intéressant de tester des prétraitements qui réduisent plutôt la cristallinité de la cellulose comme le broyage ou les liquides ioniques. En effet, il a été mis en évidence que la cristallinité de la cellulose influait négativement la production de méthane. Pour finir, et dans un but de respect de l'environnement, il serait avantageux de considérer les prétraitements fongiques qui peuvent aussi sécréter des enzymes capables de délignifier les matrices lignocellulosiques tels que la laccase, la manganèse peroxidase et la lignine peroxidase (Wan et Li, 2012).

Enfin, concernant le couplage H_2/CH_4, il serait intéressant de l'appliquer à d'autres types de substrats lignocellulosiques présentant des teneurs en sucres solubles importantes comme les bulbes ou les tiges de topinambour, ou encore tout les types de sorghos sucriers. Il serait aussi avantageux de tester des prétraitements qui permettent d'améliorer le potentiel hydrogène comme des prétraitements enzymatiques ou le couplage alcalin-enzymatique.

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ABBREVIATIONS

A: Ash

ADF: Acid Detergent Fiber

ADN: Acid Dub Nucleic

AFEX: Ammonia Fiber Explosion

Am: Amorphous holocelluloses

ARP: Ammonia Recycle Percolation

Bo: Maximum producible methane volume

BHP: Biochemical Hydrogen Potential

BMP: Biochemical Methane Potential

Carb: Carbohydrates

Cell: Cellulose

CE-SSCP: Capillary Electrophoresis-single Strand Conformation Polymorphism

COD: Chemical Oxygen Demand

Cp: Water specific heat

Cri: Crystalline Cellulose

CrI: Crystallinity Index

DP: Degree of Polymerization

DRX: X- Ray Diffraction

FT-IR: Fourier Transformed-Infared Spectroscopy

Hem: Hemicelluloses

HER: Heat Energy Requirement

HMF: Hydroxylmethylfurfural

HPLC: High Performance Liquid Chromatography

HRT: Hydraulic Retention Time

k: 1^{st} order kinetic constant

LHW: Liquid Hot Water

Lig: Lignin

LiP: Lignin peroxidise

LOI: Lateral Order Indice

m: mass

MnP: Manganese Peroxidase

Mw: Molecular weight

OLR: Organic Loading Rate

PCR: Polymerase Chain Reaction

PLS: Partial Least Square

Pro: Protein

Rm: maximum hydrogen production rate

RMSEPc: Root Mean Square Error of calibration

RMSEPv: Root Mean Square Error of validation

RNA: Rubonucleic Acid

SA: Accessible Surface

SolSu: Soluble Sugars

T : Temperature

TS: Total Solids

Ua: Uronic acid

v/v: volume/volume

VFA: Volatile Fatty Acid

Vp: Pore Volume

VS: Volatil Solids

w/v: weight/volume

w/w: mass/mass

λ: lag-phase time

General introduction

Today, fossil fuels represent the 80 % of the primary energy consumed in the world and contribute to many environmental damages, the main being global warming (Saidur et al., 2011, Nigam and Singh, 2010). In 1998, the Kyoto Protocol fixed the objective to reduce by 5.2% the world greenhouse gas emissions from the 1990 level over the 2008-2012 period. To reach this target, the use of renewable energy sources (wind, solar, hydraulic, biomass...) has been investigated. Among renewable sources, the development of technologies for producing biofuels from biomass could help to reduce the world's dependency on oils and reduce the global emissions in greenhouse gases (Naik et al., 2010).

Among biofuels, hydrogen and methane or a mixture of them, so-called "hythane", are both considered as very promising alternatives to fossil fuels (Kaparaju et al., 2009; Cavinato et al., 2010). Methane is a versatile energy vector because it can be used directly as fuel but also to produce heat and electricity through cogeneration. Compared to most liquid biofuels, biomethane exhibits far better performances with regard to both agricultural land area efficiency and life cycle emissions (Borjesson and Mattiasson, 2008). Biohydrogen can be used in internal combustion engines but also in fuel cells to produce electricity with higher efficiency. The main advantages of using hydrogen as biofuel are the absence of CO_2 emissions, its high energy content and its combustion kinetics (Koroneos et al., 2004). An alternative usage of hydrogen gas consists in using a mixture of hydrogen (5-20%) and methane (80-95%), so called "hythane". As a fuel in internal combustion engines, hythane offers several advantages compared to pure methane. In fact, since the hydrogen has a flame speed eight times higher than that of methane, the addition of hydrogen to methane results in a more stable combustion and in a lower exhaust gases emissions level than that obtained by using methane alone (Sierens and Rosseel, 2000). Porpatham et al. (2007) studied the advantages of using hythane in combustion engines and found that the addition of 10 % of hydrogen in biogas enhanced significantly the combustion, with a subsequent improvement of thermal efficiency.

Biohydrogen and methane can be produced trough anaerobic fermentative processes respectively called dark fermentation and anaerobic digestion (Figure 1). Anaerobic digestion is a biological conversion process occurring in four steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) in which the biomass is transformed into biogas, a mixture of methane and carbon dioxide. The process can also be oriented towards dark fermentation, where H_2 and CO_2 are produced instead of CH_4, by controlling the operational parameters in the reactor such as the pH and the hydraulic retention time and by inhibiting the

methanogenesis step (Hawkes et al., 2007; Nath and Das, 2004). Dark fermentation residues contain high concentration of volatile fatty acids (VFAs) (mainly acetic and butyric acids) and other compounds that can be converted in a two-stage process to methane by anaerobic digestion (Hawkes et al., 2007, Pakarinen et al., 2009). Fermentative processes such as dark fermentation and anaerobic digestion present both several advantages as they can convert a large range of feedstock using microbial mixed cultures and do not require sterile conditions to be operated. The residue of the anaerobic digestion process, called digestate, is mainly composed of stabilised organic materials which are enriched in nitrogen and phosphorus (Frigon and Guiot, 2010). The digestate can thus be used as an environmentally-friendly fertiliser for the growth of agricultural plants (Figure 1).

Biofuels, such as biohydrogen and methane produced from biomass, are classified into three categories so-called first, second and third generation fuels according to the origin of the biomass and the technologies used for their production (Nigam and Singh, 2010). The first generation biofuels are produced from the edible part of the plant (sugars, grains and seeds) while the second generation ones come from the non edible part of the plants and the third generation from microalgae (Nigam and Singh, 2010). Nowadays, the first generation biofuels are commercially available, with almost 50 billion liters produced annually worldwide. However, its production is controversial due to the competitive aspect of land usage, creating a food versus fuel dilemma (Naik et al., 2010). Thus, the second generation biofuels such as biohydrogen and methane from lignocellulosic substrates appear to be promising alternative to fossil fuels (Figure 1) (Frigon and Guiot, 2010; Guo et al., 2010a; Kaparaju et al., 2009). Moreover, methane or hythane of second generation such as others generations present zero-CO_2 emissions as CO_2 generated by the combustion can be further re-used for the growth of lignocellulosic materials during the photosynthesis (Figure 1).

The use of lignocellulosic biomass as a source of bioenergy, and especially agricultural residues as non edible residues of food crops is interesting due to its renewability and the fact that it does not create competition for lands used for food production (Ohman et al., 2006; Kleinert and Barth, 2008). Moreover, the lignocellulosic biomass is abundant as the total amount of biomass on earth is approximately 100 billion tones organic dry matter (Naik et al., 2010).

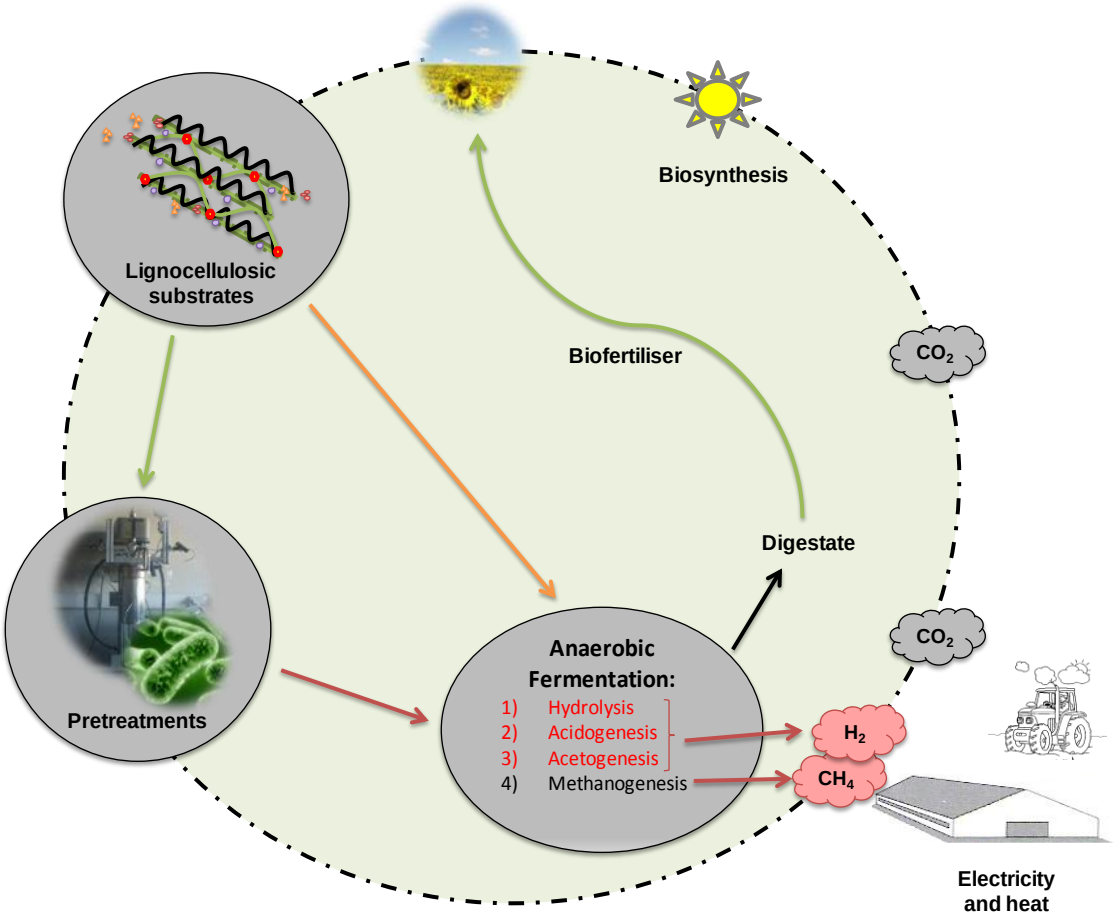

Figure 1 *Strategy of second generation biohydrogen and biomethane in integrated lignocellulosic biomass production.*

Lignocellulosic biomass such as agricultural residues consists of holocelluloses (cellulose, hemicelluloses) and lignins, which vary quantitatively and qualitatively according to the plant origin (Aman, 1993). Holocelluloses, that represent the main component of most lignocellulosic materials, have been widely described as anaerobically biodegradable in their independant form. However, several compositional and structural features can limit the microbial conversion of holocelluloses. A pretreatment step is often required to overcome these limiting factors and further enhance hydrogen and/or methane production (Chang and Holtzapple, 2000; Taherzadeh and Karimi, 2008). Many types of pretreatment have been investigated for the production of second generation bioethanol and several review papers have been published (Demirbas, 2005; Galbe and Zacchi, 2007; Galbe and Zacchi, 2002; Kumar et al., 2009a; Mosier et al., 2005; Sun and Cheng, 2002). These pretreatments are generally divided into three categories: physical (milling, irradiation, microwaves, steam explosion, liquid hot water...), thermo-chemical (alkali,

dilute acid, oxidizing agents, ionic liquid, organosolv, AFEX, wet oxidation...) and biological (fungi or enzyme)

With the aim of further industrial application, structural and compositional features that limit biohydrogen and methane production have to be well identified to develop innovative pretreatment strategies. These pretreatments strategies are highly dependent of the final end-products (ethanol, hydrogen or methane). In the case of bioethanol production; the key parameter is to convert cellulose into fermentable soluble sugars. For this purpose, combining alkaline or oxidative pretreatments and enzymatic hydrolysis or dilute-acid pretreatments are efficient to enhance the bioethanol production yield from lignocellulosic residues (Rabelo et al., 2008; Sun et al., 2005a). Nevertheless, nowadays there are limited informations about the compositional and structural characteristics that affect both the biohydrogen and methane production from lignocellulosic substrates (Guo et al., 2012; Gunasselan et al., 2009; Gunaseelan et al., 2007; Buffière et al. 2006; Triolo et al., 2011). Therefore, pretreatments strategies for increasing biohydrogen and methane production yields have not been well defined until now and various categories of pretreatments have been investigated with more or less success (Taherzadeh and Karimi, 2008, Monlau et al., 2012b). Once the pretreatment strategies will be defined, the optimization of the operational conditions of the pretreatment step shall be carried out to reduce the cost of the process and make it viable at industrial scale. Indeed, economic models show that pretreatment is an essential operation, requiring a dedicated unit accounting for more than 16-19 % of the total cost equipment of a lignocellulosic biorefinery (Aden et al., 2002).

In this context, the main objective of the present PhD thesis is to investigate the compositional and structural characteristics that limit the conversion of lignocellulosic materials into biohydrogen and methane, to further develop pretreatments strategies to enhance biohydrogen and methane production yields.

This objective was addressed by considering several scientific questions (Figure 2):

1) What are the compositional and structural features of lignocellulosic substrates that limit their conversion into biohydrogen and methane?

2) What are the impacts of thermo-chemical, enzymatic and combined chemical-enzymatic pretreatments methods on the lignocellulosic matrix?

3) How compositional and structural changes during pretreatment can affect biohydrogen production by dark fermentation?

4) How compositional and structural changes during pretreatment can affect methane production by anaerobic digestion?

5) The applicability and implementation of such pretreatment in continuous systems is also an important question for further development at industrial scale. In particular, energetic and economic balances have to be considered.

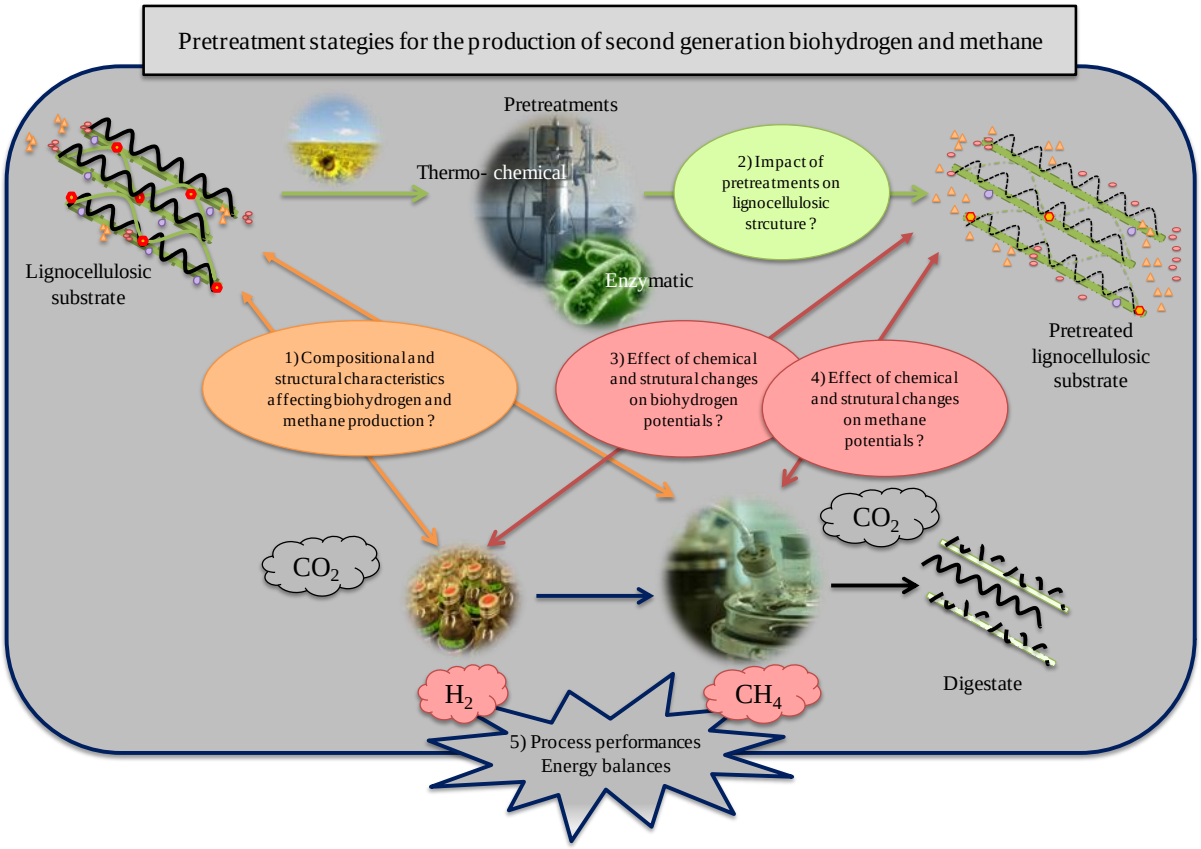

Figure 2 *Overall scheme of the main objective and scientific questions of the thesis.*

This thesis is composed of eight chapters, structured as follows:

The chapter I, entitled "litterature review", describes the scientific context of the work. It consists of a state of art on the use of pretreatments to enhance hydrogen and methane production from lignocellulosic residues. The first part describes the compositional and structural features of lignocellulosic substrates. Then, different categories of pretreatments are discussed. The last part shows how pretreatments, currently used in bioethanol processes, can be used as a tool to enhance biohydrogen or methane.

The chapter II, entitled "Materials and Methods", describes the different experimental protocols used during this work and the statistical analysis performed on the data acquired.

The results and the discussions of the thesis are included in five chapters namely III, IV, V, VI and VII. Chapter III investigates the effect of compositional and structural features of twenty lignocellulosic susbtrates on the conversion into biohydrogen and methane (question 1). Chapter IV investigates the impacts of various thermo-chemical, enzymatic or combination of these techniques on the composition and structure of lignocellulosic materials (question 2). The effect of compositional and structural changes caused by thermo-chemical, enzymatic pretreatments or combination of these on biohydrogen and methane production is discussed in chapter V and VI, respectively (question 3 and 4). At last, the best pretreatment condition defined previously for methane production in batch assay was tested in anaerobic mesophilic continuous bioreactors, to simulate the conditions in full scale anaerobic digestion plant. Two-stage H_2 (batch) / CH_4 (continuous) compared to one-stage CH_4 was also investigated. These results are presented in chapter VII (question 5).

The last chapter (VIII) corresponds to an overall conclusion of the work and proposes several perspectives for further research work.

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

● Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrere, H., Lignocellulosic materials into biohydrogen and biomethane: impact of structural features and pretreatment. Critical Reviews in Environmental Science and Technology. 2011. in press doi:10.1080/10643389.2011.604258.

● Monlau, F., Barakat, A., Steyer, J.P. and Carrere, H., Comparison of seven types of thermo-chemical pretreatment on the structural features and anaerobic digestion of sunflower stalks. Bioresource Technology, 2012, 120, 241-247.

● Monlau, F., Sambusiti C., Barakat, A., Guo, X. M., Latrille, E., Trably, E., Steyer, J.P. and Carrere, H., Modelling of biohydrogen and biomethane potential from lignocellulosic residues: effect of structural features, Environmental Science and Technology, 2012, 46, 12217-12225.

● Monlau, F., Aemig, Q., Trably, E., Steyer, J.P. and Carrere, H., Dilute-acid pretreatment of lignocellulosic biomass generate inhibitors of fermentative biohydrogen pathways, International Journal of Hydrogen, submitted.

● Monlau, F., Bonnafous, A., Trably, E., Barakat, A., Steyer, J.P. and Carrere, H., Combined thermo-alkaline and enzymatic pretreatment to enhance biohydrogen of sunflower stalks, in preparation.

● Monlau, F., Aemig, Q., Barakat, A., Steyer, J.P. and Carrere, H., Optimization of alkaline pretreatment to enhance anaerobic digestion of four varieties of sunflower stalks, in preparation.

● Monlau, F., Kaparaju, P., Trably, E., Koszela, N., E., Steyer, J.P. and Carrere, H., Alkaline pretreatment to enhance one stage CH_4 and two stages H_2/CH_4 production, in preparation.

The results obtained in this thesis have also been presented in national and international conferences (the speaker is in bold characters):

● **Carrere, H.**, Monlau, F., Barakat, A., Dumas, C. and Steyer, J.P.,Biogas from lignocellulosic biomass: Interest of pretreatment, *Progress in Biogas*, 2011, Stuttgart-Hohenheim (Germany).

● **Monlau, F**., Barakat, A., Steyer, J.P. and Carrere, H., Prétraitements de la biomasse lignocellulosiques, French Biotechnolgy School for the waste and water treatment (Narbonne 6-10 Juin 2011).

● Monlau, F., Barakat, A., **Dumas, C.**, Steyer, J.P. and Carrere, H., Impact of chemical composition and structural features on methane potential of lignocellulosic substrates, *Thirteenth International Waste Management and Landfill Symposium* , 2011, Cagliari (Sardinia).

● **Monlau, F**., Barakat, A., Latrille, E., Steyer, J.P. and Carrere, H., Impact of chemical pretreatments on solubilisation and methane production of sunflower stalks, *International Symposium on Anaerobic Digestion of Solid Waste and Energy Crops*, 2011, Vienna (Austria).

Chapter I. Litterature review

Adapted from "Monlau et al., 2012, Lignocellulosic materials into Biohydrogen and Biomethane: impact of structural features and pretreatments, Critical Review in Science and Environment, accepted"

In this chapter, a comprehensive literature review on the application of pretreatment technologies to enhance both hydrogen and methane production through lignocellulosic residues is presented. First a global vision of the world marketed energy consumption is presented with a special focus on bioenergies from biomass such as lignocellulosic residues. After, lignocellulosic residues are defined according their structural and compositional features that limit the accessibility to enzymes or micro-organisms. Then, the up-to-date available data dealing with the potential in hydrogen and/or methane production from lignocellulosic residues are presented. To finish, a focus is made on the use of different pretreatments categories to modify compositional and structural features of lignocellulosic substrates and further enhance hydrogen and/or methane production.

1. Biohydrogen and/or methane

In this subchapter, the main world marketed energy (fossil and renewable) is presented and more specifically renewable bioenergies coming from biomass known as 1st, 2nd and 3th generations biofuels. Then, a specific focus is made on biohydrogen and methane production using anaerobic fermentative processes.

1.1 Generals remarks on fuels

Currently, fossil fuels (coal, oil and natural gas) are the prime energy sources in the world corresponding to approximately 80% of the total energy use (Figure I.1), with 58% that are consumed by the transport sector (Escobar et al., 2009; Saidur et al., 2011). Fossil fuels combustion is responsible for the emission of excessive amounts of pollutants in the atmosphere, including greenhouse gases (GHG) (Escobar et al., 2009). Greenhouse gas emissions, especially carbon dioxide and methane are responsible for several environmental damages such as global warming, acid rains, rise of water level in seas, receding of glaciers, urban smog, loss of biodiversity, etc. (Nigam and Singh, 2010; Saidur et al., 2011). Moreover, the constant increase in Earth's average temperature, expected to reach from 1.4 to 5.8 °C over the period from 1990 to 2100, threatens millions of people with the growing risk of hunger, floods, water shortage and diseases such as malaria (Escobar et al., 2009). Consequently, the development of renewable sources of energy such

as wind, solar, hydraulic, biomass etc has recently generated considerable interest due to shortage of fossil fuels, increasing crude oil prices, energy security and global warming besides all the other reasons cited previously. Indeed, in 1998, the Kyoto Protocol fixed the objective to reduce by 5.2% the world greenhouse gas emissions from the 1990 level over the 2008-2012 periods. Currently, fossil fuels shift towards various renewable alternatives less environmentally harmful that account for 20% of the total world marketed energy consumption (Figure I.1).

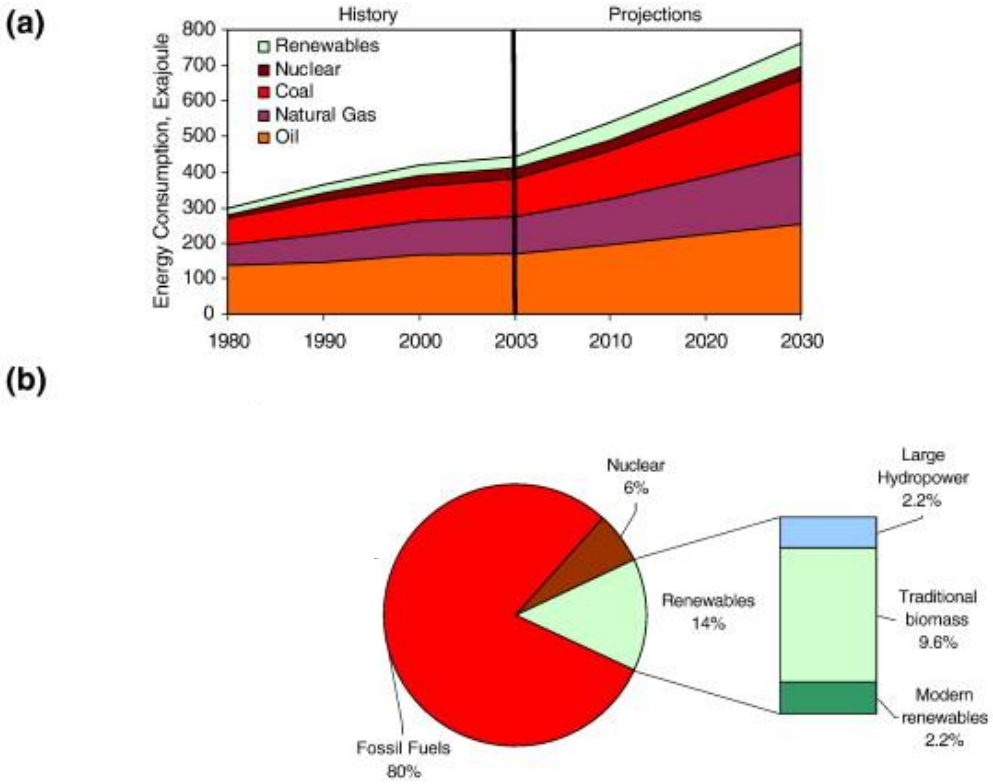

Figure I. 1 *World marketed energy consumption (a) Different fuels contribution to total world energy consumption (b) (Khan et al., 2009; Saidur et al., 2011).*

Among renewable energy vectors, the term "biofuels" are referred to liquid, gas and solid fuels predominantly produced from biomass (Nigam and Singh, 2010). The European council fixed in March 2007 the objective of 10 % minimum of biofuels to be used in the transportation sector by 2020 (EU directive, 2009). Biofuels are commonly classified in two categories from primary and secondary usages (Figure I.2). Primary biofuels are used in unprocessed form like fuelwoods, wood chips and pellets, etc (Nigam and Singh, 2010). In addition, secondary biofuels are produced after biomass processing and

transformation to ethanol, methanol biodiesel, Fisher-Tropsch diesel, hydrogen and methane and are able to be used in vehicles and various industrial processes (Nigam and Singh, 2010, Demirbas et al., 2008; Dragone et al., 2010).

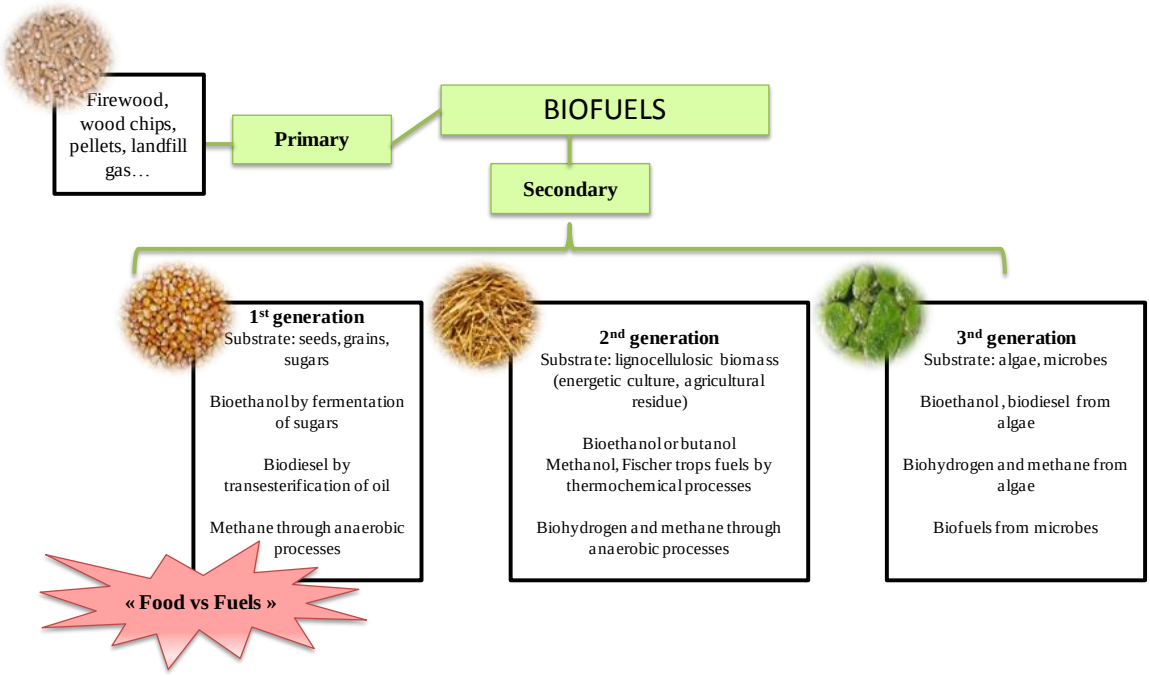

Figure I. 2 *Biofuels classification and their potentials benefits adapted from Nigam and Singh (2010) and Demirbas et al. (2008).*

Secondary biofuels can be classified into three categories (1st, 2nd and 3th generations) according to the origin of the biomass, the technologies used for their production and their level of development (Nigam and Singh, 2010) (Figure I.2). First generation bioenergies are made from agricultural feedstocks traditionally destined for food and animal purposes (IEA, 2008). These feedstocks are generally further fermented into bioethanol (rich carbohydrates crops), biodiesel (rich lipid crops) or biomethane (IEA, 2008). First generation biofuels are produced in significant amount in numerous countries such as bioethanol from sugarcane in Brazil or from maize in the United States (Nigam and Singh, 2010). However, one disadvantage of using the 1st generation biofuels is that they require significant amount of fossil fuels and fertilizers for their own cultivation (IEA, 2008). On the other hand, the production of 1st generation biofuels needs the use of arable land and thus competes with food, increasing price and lowering availability, thus raising the "food vs fuel" dilemma (Chen and Khanna, 2012). The rise in food commodity prices since

2004, which reached high record in 2008, has coincided with the tripling of corn ethanol production from 15 billion liters (BL) to 50 BL over the 2004–2010 period (Chen and Khanna, 2012). Thus, the rapid growth of biofuel production has recently become controversial. All these negative aspects have questioned their ability to replace fossil energy, and criticized their potential contribution to monoculture and deforestation (Mitchell, 2008). The combined impacts of these effects have stimulated greater interest, and even some sense of urgency, for the development of second-generation biofuels. Second generation biofuels are mainly produced from lignocellulosic residues that comprise non-edible part of the food crops production (agricultural residue) and non-edible whole plant biomass growing in general on non arable lands (energy crops).

Despite increased interest in expanding second-generation biofuels and progress made in recent years, significant bottlenecks still need to be overcome before second-generation biofuels can be produced at commercial scale, even with the massive investments in R&D observed in recent years (IEA 2008). Cellulosic ethanol is the most developed second-generation biofuel and is produced from the cellulose of plant. The main advantage of second-generation biofuels from non-edible feedstock is that it limits the direct "food versus fuel" competition (Nigam and Singh, 2010). Moreover, the second generation biofuels require more sophisticated equipment and more investment per unit of production compared to first generation biofuels (IEA, 2004).

Recently, the third generation biofuels derived from microalgae or microbes have gained consideration. These biofuels are considered to be a viable alternative energy resource with no competition with arable lands (Dragone et al., 2010; Nigam and Singh, 2010). Microalgae are able to produce 15–300 times more oil per acre than traditional crops (Schenk et al., 2008). Furthermore, comparing to the conventional crop plants which are usually harvested once or twice a year, microalgae have a very short harvesting cycle (≈1–10 days depending on the process), allowing multiple or continuous harvest with significantly increased yields (Schenk et al., 2008). However, many technical and scientific barriers such as microalgal yield, microalgal biomass harvesting, drying and processing remain to be overcome before planning a large scale production and effective commercial energy-use market of the microalgae (Dragone et al., 2010). Third generation biofuels comprise also biofuels produced from microbes. Indeed, recent studies showed that

microbial species like yeast, fungi and microalgae can biosynthesize and store fatty acids for further use as biodiesel (Zhu et al., 2008b).

This thesis focuses on second generation biofuels and specifically biohydrogen and methane. In the rest of this sub-chapter, a specific focus on hydrogen and methane production through anaerobic fermentative processes will be presented.

1.2 Biohydrogen and/or methane

Hydrogen (H_2) and methane (CH_4) are both valuable energy vectors which can be used for the production of heat and electricity or as vehicle fuel. Hydrogen is regarded as an ideal type of renewable energy for the future because it can be converted either to electrical energy in fuel cells or burnt and converted to mechanical energy without producing CO_2 (Momirlan and Veziroglu, 2005). Over the past ten years, several studies have focused on the production of biohydrogen and biomethane using lignocellulosic residues which constitute a sustainable source thanks to their abundance and low cost (de Vrije et al., 2002; Panagiotopoulos et al., 2009). Both hydrogen and methane can be produced through fermentative process. Indeed, methane can be produced from organic matter by a biological process known as anaerobic digestion while biohydrogen can be obtained by dark fermentation as a part of an anaerobic process. Indeed, the dark fermentation process is defined as an intermediate stage along the anaerobic digestion pathway when the last step (methanogenesis) does not occur or is inhibited (Figure I.3). One of the major advantages of dark fermentation and anaerobic digestion processes is that all the organic compounds can be transformed into biofuel except lignin, instead of simple sugars for bioethanol and lipids for biodiesel (Frigon et al., 2008; Xiao et al., 2010). Consequently, hydrogen or methane production in a one-stage process or combined hydrogen and methane production in a two-stage process appears to be very promising (Pakarinen et al., 2009).

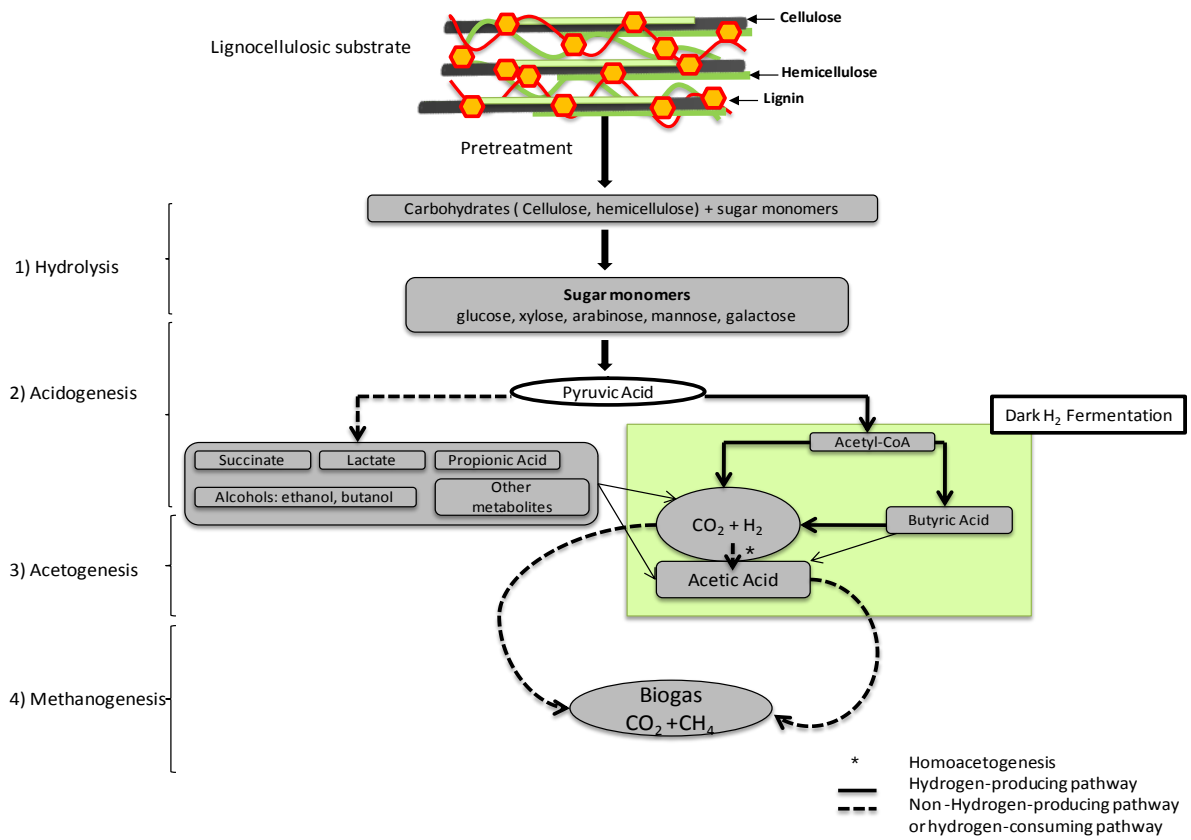

Figure I. 3 *Principle of dark fermentation and anaerobic digestion adapted to lignocellulosic biomass.*

1.2.1 Biohydrogen through dark fermentation

Biohydrogen can be biologically produced by bacterial fermentation (dark fermentation and photo-fermentation) or by a photosynthetic process carried out by microalgae (direct or indirect biophotolysis). One of the advantages in the use of the fermentation process rather photo-fermentation is that it performs concommitantly waste treatment and H_2 production (Saratale et al., 2008). In addition, dark fermentation requires less space and is around 340 times cheaper than photosynthetic production because anaerobic fermentative bacteria produce hydrogen without photoenergy (Atif et al., 2005).

Dark H_2 fermentation is a simple process that requires low energy and can use various kinds of organic waste (Wang et al., 2008). Monosaccharides (*i.e* glucose, xylose, arabinose...) and also polymers such as starch, cellulose or hemicelluloses can be used as hydrogen feedstocks. There are two common pathways in the production of hydrogen by dark H_2 fermentation: one producing acetate and the second butyrate. These

acidification processes are described by the following reactions (Equations I-1/4), using glucose and xylose as models.

The theoretical metabolic pathways of acetic acid (Equation I-1) and butyric acid (Equation I-2) from glucose are as follows (Antonopoulou et al., 2006):

$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4H_2$ (Equation I-1)

$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2\ CO_2 + 2H_2$ (Equation I-2)

The theoretical metabolic pathways of acetic acid (Equation I-3) and butyric acid (Equation I-4) from xylose are as follows (Kongjan et al., 2009a):

$C_5H_{10}O_5 + 1.67\ H_2O \rightarrow 1.67\ CH_3COOH + 1.67\ CO_2 + 3.33\ H_2$ (Equation I-3)

$C_5H_{10}O_5 \rightarrow 0.83\ CH_3CH_2CH_2COOH + 1.67\ CO_2 + 1.67\ H_2$ (Equation I-4)

Theoretically, using pure cultures, 4 moles of hydrogen can be produced from 1 mole of glucose (498 NL H_2 kg^{-1} glucose) by the acetate pathway and 2 moles (249 NL H_2 kg^{-1} glucose) by the butyrate pathway; 3.33 moles of hydrogen can be produced from 1 mole of xylose (490 NL H_2 kg^{-1} xylose) by the acetate pathway and 1.67 moles (250 NL H_2 kg^{-1} xylose) by the butyrate pathway (Antonopoulou et al., 2006; Kongjan et al., 2009a). Therefore, the butyrate / acetate ratio might be a quantitative indicator of substrate metabolism such that more hydrogen production is expected if more acetate and less butyrate are found in the system (Hawkes et al., 2007).

Among the heterotrophic bacteria that can be used for dark H_2 fermentation, anaerobic (*Clostridium*) or facultative (*Enterobacter and Bacillus*) bacteria are the most efficient microorganisms. They can be found in pure, mixed or co-cultures. Pure cultures of selected hydrogen species that include strict anaerobes (*Clostridia, rumen bacteria, archaea…*) or facultative anaerobes (*E.Coli, Enterobacter, Citrobacter…*) are often used to produce hydrogen (Ntaikou et al., 2010). Among the hydrogen-producing bacteria, *Clostridium sp*. and *Enterobacter sp* are the most widely-studied bacterial species. Hydrogen production is about 2 mol H_2/ mol glucose by *Clostridium sp.* compared to 1 mol H_2/ mol glucose by *Enterobacter sp*.

(Girbal et al., 1995; Yokoi et al., 1995). Hydrogen production using *Clostridium thermocellum* on lignocellulosic substrates has been investigated by Levin et al. (2006). A hydrogen yield of 1.6 mol H_2 mol^{-1} glucose was observed from wood fibers (Levin et al., 2006). Thermophilic biohydrogen production from energy crops by *Caldicellulosiruptor saccharolyticus* was also studied by Ivanova et al. (2009). Wheat straw was found to be the best, with a H_2 production capacity of 2.09 mol H_2 kg^{-1} TS (Ivanova et al., 2009). Co-cultures can be a promising alternative (Yokoi et al., 2001). Wang et al, (2008) investigated biohydrogen production from microcrystalline cellulose using *Clostridium acetobutylicum* in a comparison with co-cultures (*Clostridium acetobutylicum* and *Ethanoligenens harbinense)*. A hydrogen yield of 3.6 mmol H_2 g^{-1} cellulose was observed using the pure culture and 8.1 mmol H_2 g^{-1} cellulose using the co-culture. *Ethanoligenens harbinense* rapidly removed the reduced sugars produced by *Clostridium acetobutylicum* through cellulose hydrolysis, resulting in improved cellulose hydrolysis and subsequent hydrogen production rates (Wang et al., 2008). However, pure cultures need to be isolated and require aseptic conditions, which significantly increases the overall cost of the process (Ntaikou et al., 2010).

Most studies have used mixed cultures originating from natural environments such as soils and anaerobic sludge to produce hydrogen (Ntaikou et al., 2010). Mixed cultures are easier to use because they are simpler to operate and a large range of feedstock can be transformed (Li and Chen, 2007). Moreover, unlike pure cultures they do not require aseptic conditions (Ntaikou et al., 2010). However, the use of mixed cultures has the disadvantage that non-hydrogen producing species such as methanogens, homoacetogens and lactic acid bacteria can be present, leading to either the generation of byproducts like propionate, ethanol and lactate that involve the consumption of hydrogen; or to no hydrogen production (Guo et al., 2010a; Ntaikou et al., 2010). Methanogens which are considered as the main hydrogen-consuming microorganisms can be inhibited by using pretreatment such as heat shock and pH control. Heat-shock treatment methods utilise the capacity of some acidogenesic bacteria (*Bacillus and Clostridium*) to sporulate at high temperatures. In general, a heat-shock treatment of 110°C for 15 min to 2 hours is applied to eradicate non-spore-forming microorganisms (*e.g. methanogenic archae*) and to select spores of acidogenic bacteria that germinate when conditions become favourable again (Argun et al., 2008; Fang et al., 2006; Lay et al., 2003). Similarly, an acid/alkali pretreatment is an alternative to heat

pretreatment. It consists in maintaining the seed microorganisms in acidic or basic conditions over a prolonged period to eradicate the methanogens that cannot survive in conditions of extreme pH (Ntaikou et al., 2010). Chemical inhibitors such as bromoethanesulfonate, acetylene and chloroform can also be used (Guo et al., 2010a). However, hydrogen potentials results are highly dependent of the nature of mixed cultures used. Indeed, for a same substrate (wheat stalk), Chu et al. (2011) have shown that hydrogen potentials varied from 23 L $H_2\,kg^{-1}$ VS to 37 L $H_2\,kg^{-1}$ VS with anaerobic digested sludge and anaerobic digested dairy manure as mixed cultures respectively. Chu et al. (2011) suggested that probably a bioaugmentation of cellulose-degrading bacteria in anaerobic digester of dairy manure is present as more cellulose in the manure needs to be degraded than that in sludge (Chu et al., 2011). Similar trends have been observed by Lay et al., (2012) on hydrogen production from mushroom cultivation waste using various anaerobic mixed cultures. Indeed, hydrogen potentials of 2.7 L H_2 kg^{-1} VS, 3.4 L H_2 kg^{-1} VS and 10.1 L H_2 kg-1 $_{VS}$ were respectively observed with sewage sludge, pig slurry and cow dung compost.

1.2.2 Methane trough anaerobic digestion

Anaerobic digestion of lignocellulosic residues consists of a complex series of metabolic interactions involving different anaerobic micro-organisms in an oxygen-free environment and leads to the formation of biogas which consists mainly of methane (55-75%) and carbon dioxide (25-45%). The process can be divided in four phases of biomass degradation and conversion, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure I.3). Each stage requires the activity of its own specific group of micro-organisms.

During the hydrolysis step, complex organic molecules (proteins, lipids and carbohydrates) are hydrolyzed into simple sugars, amino acids, and fatty acids. Hydrolytic bacteria (primary fermenting bacteria) are facultative anaerobes and they hydrolyze the substrate with extracellular enzymes. A wide range of enzymes (cellulases, hemicellulases, proteases, amylases, lipases) can be produced during the biogas process and thus, biogas processes can hydrolyze almost all kinds of substrates (Taherzadeh and Karimi, 2008). When the microorganisms can produce the suitable enzymes, hydrolysis is a relatively fast step and become rate-limiting if the substrate is hardly accessible for the enzymes (Taherzadeh and Karimi, 2008).

During acidogenesis, primary fermenting bacteria absorb the products of hydrolysis and convert them into volatile fatty acids (VFA), hydrogen and alcohols. The microorganisms involved are both obligate and facultative anaerobes. During optimal conditions, primary fermentative bacteria produce mainly acetic acid, hydrogen and carbon dioxide and these can be used directly as substrates by the methanogenic microorganisms. On the contrary, if the environmental conditions are not optimal (high organic loading rate or presence of toxic compounds), this pathway is not favorable and the primary fermenting bacteria switch metabolism, producing other intermediates (Klass, 1984), such as VFA longer than two carbon atoms and alcohols longer than one carbon atom (Bryant, 1979; Schink, 1997). Methanogenic microorganisms cannot use directly these reduced intermediates, therefore these products have to be further modified, during acetogenic phase, before they can be converted into biogas.

During acetogenesis takes place the conversion of the acidogenic products (mainly VFA) into acetic acid, hydrogen and carbon dioxide. This phase is carried out by secondary fermenting bacteria. These microorganisms are obligate hydrogen-producing bacteria

Finally, methanogenesis is the conversion of acetate, CO_2 and H_2 to methane by archae microorganisms. The mixture CO_2/H_2 is transformed into methane by hydrogenophilic methanogens while acetate is transformed into methane by acetoclastic methanogens. Thus, the final product of anaerobic digestion is biogas which consists mainly of methane (55-75%) and CO_2 (25-45%).

As anaerobic digestion is a biological process, it is strongly influenced by the following environmental factors: temperature, pH, and toxic compounds. Anaerobic digestion is divided into psychrophilic (10-20°C), mesophilic (20-40°C) or thermophilic (50-60°C) digestion processes. The first stage in anaerobic digestion can occur at a wide range of pH whereas methanogenic micro-organisms are efficient only at neutral pH (6.5-7.5). At excessive concentrations, such compounds as VFA, hydrogen, ammonia or heavy metals may affect methanogenesis.

1.2.3 Coupling bioH_2 and CH_4 production

Only about 10-20% of the energy potential of an organic substrate is obtained through dark hydrogen fermentation (Cooney et al., 2007). Dark fermentation residues contain VFA (mainly acetic and butyric acids) and other compounds which will not be degraded to H_2 due to thermodynamic restrictions (Hawkes

et al., 2007). There are several routes for using such residues in a second stage; these include converting the by-products to H_2 using photosynthetic bacteria or to electricity using Microbial Fuel Cells or to CH_4 during an anaerobic process (Ren et al., 2009, Martinez-Pérez et al., 2007; Oh and Logan, 2005).

In the photofermentation process, acetate and butyrate derived from soluble metabolites of the dark fermentation can be converted into hydrogen by photosynthetic bacteria, known for their dominant tendency to convert organic acids to hydrogen in the presence of light, and by the action of the nitrogenase enzyme (Claassen et al., 2004). The combination of dark and photo fermentation can achieve a theoretical maximum hydrogen yield of 12 mol H_2 mol^{-1} hexose. This kind of two-stage bioprocess has been investigated using lignocellulosic substrates such as potato steam peel and cassava starch (Claassen et al., 2004; Su et al., 2009). By a combination of dark and photo fermentation, the maximum hydrogen yield from cassava starch increased from 240 L H_2 kg^{-1} starch to 402 L H_2 kg^{-1} starch compared to dark fermentation alone (Su et al., 2009). However, one of the major drawbacks of this process is its costs because photo-heterotrophic bacteria employ light as their primary energy source and organic compounds as the carbon source (Claassen et al., 2004).

Another possibility is to convert VFAs generated during dark H_2 fermentation into electricity through Microbial Fuel Cells (Oh and Logan, 2005). Oh and Logan (2005) have shown that end-products from batch hydrogen production of cereal processing wastewater can be further used to produce electrictity by Microbial Fuel Cells with 95 % of COD removal. Byproducts from dark H_2 fermentation can be also further converted into methane through a two-stage H_2/CH_4 process (Figure I.4).

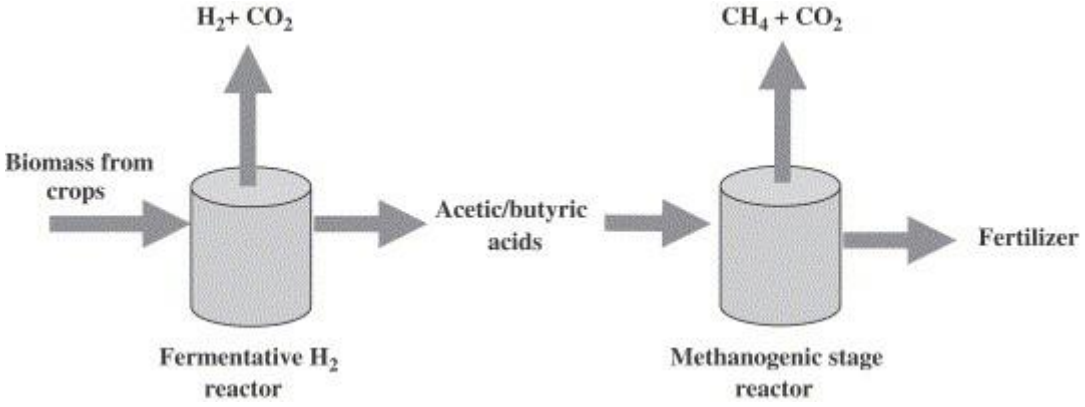

Figure I. 4 *Two-stage system for hydrogen and methane production from wet biomass (Martinez-Pérez et al., (2007).*

In the first stage, the operating conditions (acid pH and hort retention time) are set to favour fermentation of the substrate to hydrogen by enhancing the growth of acidogenic bacteria. In the second stage, conditions are changed to suit methanogenesis (neutral pH and longer retention time). This kind of process presents several advantages because the first stage acts efficiently for solubilization and the combined hydrogen-methane mixture called "hythane" has been shown to burn cleaner than methane alone (Bauer and Forest, 2001; Ueno et al., 2007). However, two-stage process in some cases did not enhance energy yield production compared to one-stage process. Indeed, Pakarinen et al., (2011) recorded no significant energy yield increase on maize between two-stage H_2/CH_4 process and one-stage CH_4 process. Moreover in the case where two-stage H_2/CH_4 process is beneficial, the energy gain from only one-stage CH_4, process should be compared to the higher investments and operational costs due to two-stage process.

2. Lignocellulosic substrates

In this sub-chapter, the chemical composition of lignocellulosic substrates is first presented and in a second part the compositional and structural characteristics that limit the microbial accessibility to biodegradable compounds of the lignocellulosic matrix are presented.

2.1 Chemical composition

Lignocelluloses in nature derive mainly from wood, grass, agricultural residue or energy crops (Table I.1). Lignocellulosic substrates are composed of three main fractions: cellulose (30-60 %), hemicelluloses (10-40 %) and lignin (5-30%) as well as minority fractions (soluble sugars, pectin and proteins) (Figure I.5). The composition of the three main fractions (cellulose, hemicelluloses and lignin) varies according one plant species to another and within the same plant, composition varies according the species, plant part and maturity (Vanholme et al., 2010). Table I.1 presents compositions of cellulose, hemicelluloses and lignin contents encountered in the most common sources of lignocellulosic biomass.

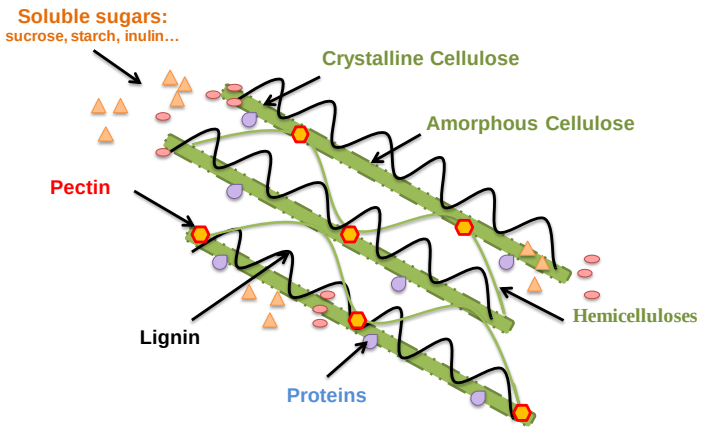

Figure I. 5 *Scheme of compositional and structural features of milled lignocellulosic substrates. Both intracellular and extracellular parts are represented.*

2.1.1 Cellulose

Cellulose consists of D-glucose subunits, linked by β-(1→4) glycosidic bonds (Fengel, 1992; Fengel and Wegener, 1984). The cellulose in a plant consists of parts with an organized crystalline structure and parts with a poorly organized amorphous structure. The cellulose strains are 'bundled' together and form so-called cellulose fibrils or cellulose bundles. These cellulose fibrils are mostly independent and weakly bound through hydrogen binding (Liang and Marchessault, 1959; Vanderhart and Atalla, 1984). Cellulose, insoluble in water and most organic solvents, is chiral and biodegradable. It can be broken down chemically into its glucose units by treating it with concentrated acids at high temperature.

2.1.2 Hemicelluloses

Hemicelluloses are polysaccharides heteropolymers present in almost all plant cell walls along with cellulose (Aman, 1993). While cellulose presents amorphous and organized crystalline parts, hemicelluloses have a random, amorphous structure with little strength. Hemicelluloses have a lower molecular weight than cellulose. They have branches with short lateral chains that consist of different sugar monomers and can include xylose, mannose, galactose and arabinose, which are polymers that can be easily hydrolysed by both dilute acid or a base as well as by numerous hemicellulase enzymes (Ebringerová and Heinze, 2000; Fengel and Wegener, 1984; Kacurakova et al., 1999). Xylose is generally the sugar monomer present in the largest amount, though uronic and ferulic acids also tend to be present. The

dominant component of hemicelluloses from hardwood and agricultural plants such as grasses and straws is xylan, while in softwoods glucomannan is dominant (Ebringerová and Heinze, 2000; Ebringerova et al., 1994; Fengel and Wegener, 1984; Sun et al., 1996). Hemicelluloses bind to cellulose with pectin to form a network of cross-linked fibers made up of hemicelluloses and lignin which represent a limiting factor in the biodegradation of lignocellulosic materials (Watanabe et al., 2003). The hemicelluloses are the most thermal-chemically sensitive of lignocellulosic components (Levan and Winandy, 1990; Winandy et al., 1991). During thermal-chemical pretreatments, it is the side groups of hemicelluloses that react first, followed by the hemicelluloses backbone.

2.1.3 Lignin

Lignin is the third most abundant polymer in nature, after cellulose and hemicelluloses, and is present in cell walls. It is an amorphous heteropolymer consisting of three different phenylpropane alcohols: p-coumaryl (H), coniferyl (G) and sinapyl (S) (Table I.1). The nature and the quantity of lignin monomers (H, G, S) vary according to species, maturity and the space localization in the cell (Yoshizawa et al., 1993). For instance, an increase in lignin content from 3% to 7% was observed during the maturing of grass (Nizami et al., 2009). There are three main groups of lignins: the lignins from softwoods (gymnosperms) contain mainly guaïacyl units, those from hardwoods (angiosperms) mainly guaïacyl and syringyl units, whereas the lignins from herbaceous plants (non-woody or gramineae) contain all three units (H, G, S) in significant amounts with different ratios (Billa and Monties, 1995; Boerjan et al., 2003; Lapierre et al., 1986; Nimz et al., 1981; Vanholme et al., 2010). The main purpose of lignin is to give the plant structural rigidity, impermeability and resistance against microbial attack and oxidative stress. The amorphous heteropolymer is also insoluble in water and optically inactive, which makes the degradation of lignin very difficult (Akin, 2008; Fengel and Wegener, 1984; Grabber, 2005). Lignin normally starts to dissolve in water at around 180°C under neutral conditions (Kubikova et al., 1996). The solubility of lignin in acid, neutral or alkaline environments depends, however, on the precursor of the lignin (p-coumaryl, coniferyl, sinapyl alcohol or combinations of them) (Grabber, 2005).

2.1.4 Other compounds: pectins, proteins and soluble sugars

Pectin which is a common constituent of fruit wastes or other residues of the food industry is also present in plant cell walls of lignocellulosic biomass. For instance, grasses contain 2-10% of pectin and wood tissues around 5% (Voragen et al., 2009). Pectin is used as a gelling agent in many industrial applications due to its ability to form gels in the presence of Ca^{2+} ions (Axelos and Thibault, 1991). Like other polymers, pectins contribute to give physical strength to the plant and to provide a barrier against the outside environment (Harholt et al., 2010). Pectin is defined as a hetero-polysaccharide containing in majority galacturonic acid and the main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan (Cosgrove et al., 2005). Homogalacturonan is the most abundant constituting about 65% of pectin content, while rhamnogalacturonan I accounts for 20% to 35% (Mohnen, 2008). Homogalacturonan is a polymer of α-1,4-linked galacturonic acid residues and the minimum length estimated is for citrus, sugar beet and apple pectin with 72-100 galacturonic acid residues (Thibault et al., 1993). According to Zykwinska et al. (2005), pectin binds to cellulose to form a network of cross-linked fibers. Pectins are wall polysaccharides that are solubilized by aqueous buffers and dilute acidic solutions or calcium chelators (Cosgrove et al., 2005).

Lignocellulosic substrates are also composed of a small amount of proteins (Cosgrove, 2005). For instance, protein contents of 3.3, 3 and 2.1 % per TS were respectively observed for maize, grass and straw (Triolo et al., 2011). Guo et al. (2012) have evaluated proteins contents of 3.3, 3.4 and 5.1 % TS respectively for rice straw, giant reed stalks and giant reed leaves.

Lignocellulosic substrates comprise sometimes a part of soluble sugars mainly starch, sucrose or inulin. Starch and sucrose are easily soluble in water and not bound to the lignocellulosic structure (Chen et al., 2007). High amount of soluble carbohydrates of 16.9 % and 28.1 % per VS were respectively observed for sweet sorghum and napiergrass (Gunaseelan et al., 2007). Sugar beets are composed of 67.3 % TS of sucrose and only 4.2 % and 5.2 % respectively of cellulose and hemicelluloses (Panagiotopoulos et al., 2009). Inulin (β 2, 1 fructose) is also an easily soluble sugar present in some lignocellulosic substrates. For instance, Jerusalem artichoke is made up of 70-90 % of inulin (Thuesombat et al., 2007).

2.2 Compositional and structural features affecting accessibility and biodegradability of lignocellulosic substrates

The mechanical, physical, chemical and biological properties of lignocellulosic materials are dependent not only on the chemical composition of the matrix but also on the organisation of their constituents and the interaction between them (Salmen and Olsson, 1998). The cell wall may be schematically viewed as cellulose microfibril bundles arranged in parallel in a matrix of amorphous hemicelluloses and lignin, as shown in Figure I.5. A considerable amount of work has been carried out to try to determine the substrate characteristics which lead to a decrease in the rate of cellulose hydrolysis and, in many cases, incomplete hydrolysis of the lignocellulosic substrates (Hendriks and Zeeman, 2009; Koullas et al., 1992; Yoshida et al., 2008; Zhu et al., 2008a). Most of this works concerned bioethanol production and focused on the separation of cellulose from lignin and hemicelluloses in order to enhance enzymatic cellulose hydrolysis. Microbial or enzymatic accessibility can be affected by different factors such as degree of polymerization and crystallinity of the cellulose, the structure of hemicelluloses, pectin content, accessible surface area and pore volume and the lignin content and composition.

2.2.1 Cellulose: crystallinity and degree of polymerisation

Hayashi et al. (2005) have suggested that during the enzymatic hydrolysis of cellulose the readily accessible regions (amorphous regions) are more efficiently hydrolyzed, resulting in an accumulation of crystalline cellulose. Crystallinity (CrI) of cellulose is commonly determined by diffraction rayon X measurement and represents the proportion of crystalline cellulose in the biomass.

Other authors have suggested that the rate of cellulose degradation decreases as a result of structural transformations during the initial stages of hydrolysis with the result that the more resistant fraction remains unhydrolyzed and as a consequence, cellulase digestibility of the treated biomass is limited by cellulose accessibility (Gupta and Lee, 2009a; Jeoh et al., 2007; Mansfield and Meder, 2003; Mooney et al., 1999). Many properties of cellulose depend on its crystallinity (CrI), molecular weight (M_w), degree of polymerization (DP), surface area, all of which depend on the species, plant part, and plant maturity (Table I.1). All these parameters have been shown to influence the enzymatic hydrolysis of cellulose. Some work

has shown a good correlation between crystallinity and the rate of enzymatic hydrolysis of cellulose (Ciolacu et al., 2008; Gupta and Lee, 2009a). However, with lignocellulosic materials this relationship is not so clear-cut, due to the more heterogeneous nature of this material and the contribution of other components such as lignin and hemicelluloses. (Chang and Holtzapple, 2000; Koullas et al., 1992)

2.2.2 Influence of hemicelluloses

In contrast to cellulose, the effect of the physicochemical properties of hemicelluloses on the accessibility of lignocellulosic substrates and their biodegradability into biogas and bioethanol has not been studied. Yet, of the total mass in the residues of annual plants which can be fermented to biogas, hemicelluloses (C5-sugars) represent about 20-40%. Hemicelluloses serve as a connection between the lignin and the cellulose fibers (Salmen and Olsson, 1998; Watanabe et al., 2003). In general, the dominant hemicelluloses from all plant cell walls are xylans (Table I.1). The structure of xylan is more complex than that of cellulose and has been fully described in several reviews on hemicelluloses in wood and grass (Izydorczyk and Dexter, 2008; Izydorczyk and MacGregor, 2000; Puls, 1997; Saake et al., 2001). Xylan structure depends on the degree of substitution of the xylose linear chains by arabinose and uronic acids and on the molar mass. All these parameters depend on the species, plant part, and plant maturity (Table I.1) (Aman, 1993; Dervilly et al., 2000; Izydorczyk, 2009; Saulnier et al., 1997; Saulnier et al., 1999). Hemicelluloses also contain smaller amounts of nonsugars such as acetyl groups that can limit enzymatic hydrolysis (Kumar et al., 2009a). Chang and Holtzapple (2000) reported negative correlations between enzymatic digestibility and acetyl contents. Moreover, Kong et al. (1992) have shown that deacetylation increases the yield of sugars obtained from enzymatic hydrolysis of aspen wood (Kong et al. 1992). Considering the chemical and structural complexity of grass hemicelluloses, it is not surprising that nature has developed a complete arsenal of hemicelluloses-hydrolyzing enzymes that, through their concerted action, bring about complete degradation of these polymers. The main depolymerising enzyme is xylanase whose action is complemented by that of arabino-hydrolyzing and acetyl esterases enzymes.

2.2.3 Influence of lignin content and composition

Major roles of lignin are to ensure impermeability, maintain fibre integrity and the structural rigidity of the plant. Lignin is a polymer of phenylpropane units which form a three-dimensional network inside the cell wall. The major inter-unit linkage is an aryl-ether type β-O-4 link. The macromolecular structure of the lignin polymer depends on the β-O-4 linkage, monomer distribution (G, S and H) and molecular weight, all of which depend on the species, plant part, and plant maturity (Fukushima and Terashima, 1991; Vanholme et al., 2010). These different parameters modify the architecture and supramolecular organization of the cell wall and influence its accessibility and digestibility (Akin et al., 1995; Chang and Holtzapple, 2000; Jung and Engels, 2002; Laureano-Perez et al., 2005; Tian et al., 2010). The enzymatic degradability of cell walls in leaves and particularly in the stems of plants declines during maturing because of progressive lignification of the cell walls tissues.

The distribution and composition of lignin are also very important for enzyme accessibility and the digestibility of biomass (Adler, 1977; Clark et al., 2009; Guo et al., 2010b; Ntaikou et al., 2010; Yuan et al., 2008). For example, these factors have been cited as being responsible for the higher recalcitrance of softwood-derived substrates (Mooney et al., 1999; Mooney et al., 1998). It has also been suggested that guaïacyl lignin restricts fiber swelling and, hence, enzyme accessibility more than syringyl lignin (Mooney et al., 1999; Mooney et al., 1998).

In an attempt to correlate substrate accessibility with the efficiency of enzymatic hydrolysis, various studies measured the initial enzyme adsorption capacity of different substrates and correlated it with the initial rates of hydrolysis. It was found that substrates containing little or no lignin showed good correlation between initial hydrolysis rates and adsorption capacity, while substrates with higher lignin content demonstrated a poor correlation (Chang and Holtzapple, 2000; Koullas et al., 1992).

2.2.4 Influence of pectin contents

Some recent works have shown that pectin content can be an important parameter on lignocellulosic conversion by limiting the enzyme accessibility to cellulose (Berlin et al., 2007; Pakirinen et al., 2012; Frigon et al., 2011). Berlin et al. (2007) suggested that the use of pectinase enzymes can increase the hydrolysis of cellulose. Indeed, Pakirinen et al. (2012b) have shown that the hydrolysis of pectin on fiber

hemp using commercial pectinase (Pectinex, Novozyme, Denmark, 2.5 mg protein g^{-1} TS substrate) can increase the enzymatic hydrolysis yield by 26 % for the theoretical carbohydrates of untreated hemp. However, on the same substrate, hot alkali treatment (121°C, 1h, 1% NaOH (w/w)) and steam explosion (14,5 bar, 200°C, 5min) were found more efficient by increasing the conversion of the total carbohydrates by 60 % and 78 % (Pakirinen et al., 2012b).

2.2.5 Surface area and pore volume

Other parameters such as pore volume and accessible surface area have been shown to affect the biodegradability of lignocellulosic materials. Several groups showed good correlations between pore volume, surface area and the enzymatic digestibility of lignocellulosic materials suggesting that increasing the surface area of a substrate enhances its digestibility (Chang and Holtzapple, 2000; Koullas et al., 1992; Laureano-Perez et al., 2005; Park et al., 2007; Puri, 1984).

Table I. 1 *Biochemical composition of different lignocellulosic biomasses.*

	Grasses and Gramineae						Energy crop	Hardwood		Softwood	
Lignocellulosic compounds	**Wheat straw**	**Wheat bran**	**Corn stover**	**Rice straw**	**Barley straw**	**Rye straw**	**Miscan-thus**	**Poplar**	**Eucalyptus**	**Spruce**	**Pine**
Celluloses (%)	**39.6**	**42.5**	**36.8**	**32.0**	**37.5**	**38.0**	**37.7**	**44.5**	**54.1**	**45.5**	**43.3**
Mw (g/mol)	250.720	-	-	272.130	337.820	241.830	-	171.950	-	-	-
DP	1547	-	7050	1680	2085	1439	-	1091	-	-	-
CrI(%)	**50.3**	-	**50.3**	**51.7**	-	-	-	**49.9**	**54.3**	**38.4**	-
Hemicelluloses (%)	**26.6**	**21.2**	**30.6**	**18.0**	**25.3**	**36.9**	**37.3**	**22.5**	**18.4**	**22.9**	**21.5**
Xylose (Xyl)	24.3	15.4	22.2	14.3	21.7	-	33.8	19.4	15.0	6.6	5.3
Arabinose (Ara)	2.1	3.1	5.5	2.3	2.5	-	2.8	0.5	0.5	1.2	1.6
Galactose	0.2	2.7	2.9	1.2	1.2	-	0.6	2.0	1.5	0.6	2.9
Mannose	-	-	-	0.2	-	-	0.1	0.6	1.5	13.5	10.7
Ara/Xyl	**0.09**	**0.20**	**0.25**	**0.16**	**0.12**	-	**0.08**	**0.025**	**0.03**	**0.18**	**0.3**
Lignin (%)	**21.0**	**3.4**	**23.1**	**11.2**	**16.0**	**17.6**	**25.1**	**19.5**	**21.5**	**27.9**	**28.3**
G /S /H	**49/46/05**	-	**35/61/4**	**45/60/15**	-	**44/54/2**	-	**41/59/nd**	**38/62/nd**	**98/tr/2**	**82/tr/18**
β-O-4 (µmol/g of lignin)	**1040**	-	**610**	**630**	-	**1610**	-	**2390**	**2780**	**1230**	**1140**
Mw (g/mol)	2800	-	-	3600	3200	3000	-	5500	≥5000	≥6000	≥8000
Others (%)	5.5	-	9.5	14.2	6.4	6.3	-	-	3.0	4.2	7.9
References	(Akpinar et al., 2009; Lapierre, 1993; Lequart et al., 1999; Sun et al., 2005a)	(Lequart et al., 1999)	(Lapierre, 1993; Sun et al., 2005a; Sun et al., 2002; Teramoto et al., 2009)	(Persson et al., 2009; Sun et al., 2005a; Sun et al., 2002; Teramoto et al., 2009)	(Akpinar et al., 2009; Gullón et al., 2009; Sun et al., 2005a)	(Aguilar et al., 2002, Sun and Cheng, 2005b)	(Brosse et al., 2009)	(Guerra et al., 2006; Lapierre, 1993; Popescu et al., 2009; Santiago and Neto, 2008; Sun et al., 2005a)	(Galbe and Zacchi, 2007; Guerra et al., 2006; Lapierre, 1993; Santiago and Neto, 2008)	(Galbe and Zacchi, 2007; Lapierre, 1993; Palm and Zacchi, 2004; Popescu et al., 2009)	(Galbe and Zacchi, 2007; Lapierre, 1993; Tejado et al., 2007)

G : Guaiacyl units in lignin ; S : Syringyl units ; H : p-Hydroxyphenyl; Mw: Molecular mass ; DP: Degree of polymerization; CrI: Crystallinity Index; nd: not detected; tr: traces

3. Conversion of lignocellulosic materials into biohydrogen and/or methane

In this sub-chapter, biohydrogen and methane potentials from lignocellulosic residues are first discussed. Then, two-stages H_2/CH_4 processes are presented.

3.1 Biohydrogen from lignocellulosic substrates

Recently, Guo et al. (2012) have investigated the hydrogen potentials from 26 organic solid wastes containing carbohydrate-rich substrates, protein-rich substrates, agri-industry waste and agricultural wastes. They found an average hydrogen production of 174 L H_2 kg^{-1} VS, 5.5 L H_2 kg^{-1} VS, 47.5 L H_2 kg^{-1} VS, 24.9 L H_2 kg^{-1} VS, respectively for carbohydrate-rich substrates, protein-rich substrates, agri-industry waste and agricultural wastes. Carbohydrates-rich substrates and in a lesser extent agricultural wastes represent preferential biomasses sources for hydrogen production than protein-rich substrates (Guo et al., 2012). Indeed, while carbohydrates and proteins are both basic components of organic materials, in terms of hydrogen yield during dark fermentation, carbohydrates are known to be a better substrate than proteins (Bai et al., 2004). The maximum hydrogen yields are about 6.25 mmol H_2 g^{-1} glucose (140 mL H_2 g^{-1} glucose) and 3.43 mmol H_2 g^{-1} protein (77 mL H_2 g^{-1} protein) at initial neutral pH (Xiao et al., 2010). However, protein can improve the cell growth of hydrogen-producing bacteria and consequently increase hydrogen productivity (Brosseau et al., 1986). For example, substrates containing 60 % glucose and 40% peptone were tested and provided better conditions for cell growth and biohydrogen production than a substrate containing only glucose (Bai et al., 2004).

Lignocellulosic materials represent interesting substrates for hydrogen production, as has been shown by extensive reviews of biohydrogen production involving a large range of substrates (Demirel et al., 2010, ; Guo et al., 2010a; Cheng et al., 2011a). Some studies using lignocellulosic feedstocks to produce biohydrogen are mentioned in Table I.2 with values ranging from 3.2 L H_2 kg^{-1} VS (corn stalk) to 106 L H_2 kg^{-1}VS (Jerusalem artichoke tubers). Substrates containing high amounts of inulin as Jerusalem artichoke stalks and tubers give also interesting hydrogen potentials with respectively 68 and 106 L H_2 kg^{-1} VS (Guo et al., 2012). Similar trends have been noticed for sorghum containing high contents of soluble carbohydrates with hydrogen potentials ranging from 30.5 L H_2 kg^{-1} VS to 68 L H_2 kg^{-1} VS. Indeed, Gunaseelan (2007) have evaluated a high soluble carbohydrate content of 23 % per VS in sorghum bicolour

roots. It seems that substrates containing high content of easily soluble sugars as sucrose, starch and inulin are more easily converted into biohydrogen as previously suggested by Guo et al. (2012) who found a correlation (R^2 =0.89) between biohydrogen accumulation and the content in soluble carbohydrates, extracted under mild acidic conditions (2 N hydrochloric acid). Prakasham et al., (2012) showed that sorghum derivatives mutants with lower lignin content give interesting hydrogen potentials of 68 L H_2 kg^{-1} VS compared to 50 L H_2 kg^{-1} VS for raw sorghum sample suggesting that lignin content plays a role on fermentative hydrogen production.

Table I. 2 *Hydrogen potentials from lignocellulosic substrates according literature data.*

Substrate	**Hydrogen yield (L H_2 kg^{-1} VS_{added})**	**Energy yield (MJ kg^{-1} VS_{added})[a]**	**References**
Corn stalk	3	0.03	(Zhang et al., 2006)
Maize stalk	3.5	0.04	(Guo et al., 2012)
Wheat straw	8	0.08	(Quemeneur et al., 2012a)
Jerusalem artichoke leaves	13	0.14	(Guo et al., 2012)
Corn-cob	13	0.14	(Pan et al., 2009)
Sunflower oil cakes	14	0.15	(Guo et al., 2012)
Poplar leaves	15	0.16	(Cui et al., 2010)
Maize leaves	17	0.14	(Ivanova et al., 2009)
Rice straw	21.5	0.23	(Guo et al., 2012)
Giant reed leaves	22	0.24	(Guo et al., 2012)
Giant reed stalks	30.5	0.33	(Guo et al., 2012)
Sweet sorghum	30.5	0.33	(Ivanova et al., 2009)
Wheat straw	46	0.5	(Ivanova et al., 2009)
Sorghum	50.5	0.54	(Prakasham et al., 2012)
Wheat bran	51	0.55	(Pan et al., 2008)
Sweet sorghum stalk	52.1	0.56	(Shi et al., 2010)
Jerusalem artichoke stalks	68	0.73	(Guo et al., 2012)
Sorghum mutants derivatives	68	0.73	(Prakasham et al., 2012)
Jerusalem artichoke tubers	106	1.14	(Guo et al., 2012)
Fodder maize	61[c]	0.66	(Kyazze et al., 2008)
Ryegrass	73[c]	0.79	(Kyazze et al., 2008)
Beet pulp	78.5[c]	0.85	(Ozkan et al., 2010)
Beer residues	3[b]	0.034[b]	(Cui et al., 2009)
Grass	4.5[b]	0.05[b]	(Cui et al., 2011)
Sweet sorghum	59[b]	0.63[b]	(Ntaikou et al., 2007)
Wheat flour	60[b]	0.65[b]	(Hawkes et al., 2008)

[a] Energy yield 1Nm^3 H_2 = 10780 kJ.
[b] L H_2 kg^{-1} TS added or MJ kg^{-1} TS added.
[c] calculated from the literature

3.2 Methane from lignocellulosic substrates

The advantage of using anaerobic digestion is that not only simple organic compounds such as pentoses, hexoses, and volatile fatty acids but also polymers, (cellulose, starch, hemicelluloses...) are converted into methane. Even inhibiting compounds of bioethanol fermentation (furfural, HMF and soluble lignin) can be transformed into methane if not highly concentrated (Benjamin et al., 1984; Barakat et al., 2012).

Based on the stoechiometric conversion of organic material into methane and carbon dioxide, the theoretical or maximum methane yield (100% conversion) can be calculated from the elemental composition of the substrate $C_aH_bO_cN_dS_e$ as follows (Frigon and Guiot, 2010; Lubken et al., 2010) :

$$Y_{CH4}^{Theoretical}\left(L/g_{substrate}\right)=\frac{22.4(4a+b-2c-3d-2e)}{8(12a+b+16c+14d+16e)} \quad \text{(Equation I-5)}$$

According to equation I-5, the theoretical methane yields of carbohydrates ($C_{10}H_{18}O_9$) and lignocellulosic biomass ($C_5H_9O_{2.5}NS_{0.025}$) are 397 NL CH_4 kg^{-1} VS and 475 NL CH_4 kg^{-1} VS, respectively (Frigon and Guiot, 2010). Based on measured elemental analysis, Lubken and al. calculated the theoretical methane yields of various form of lignocellulosic biomass. Results ranged from 394 NL CH_4 kg^{-1} VS for triticale straw to 0.490 NL CH_4 kg^{-1} VS for rye grass (Lubken et al., 2010). However, actual methane yields from lignocellulosic biomass can be far lower than the theoretical amounts. Several extensive reviews of methane production from biomass have been published (Frigon and Guiot, 2010; Ward et al., 2008). Some results are presented in Table I.3 where values range from 97 L CH_4 kg^{-1} VS (Newsprint) to 431 L CH_4 kg^{-1} VS (grass sillage).

It is generally accepted that the higher the crude fiber content, the lower the methane potential of the biomass. This has been confirmed on three types of paper (newsprint, paper tube residues and office paper) with different lignin content (30.3%, 23%, 3.6% respectively) which had a methane potential of 97 L CH_4 kg^{-1} VS_{added}, 222 L CH_4 kg^{-1} VS_{added}, 364 L CH_4 kg^{-1} VS_{added}, respectively (Teghammar et al., 2009; Xiao et al., 2010). According to Kobayashi et al (2004), a linear regression shows a strong negative correlation between the amount of methane produced and the amount of Klason lignin in bamboo. The lignin content plays a major role in methane production by limiting the access to holocelluloses. Holocelluloses, which are anaerobically-biodegradable compounds when they are alone, appear to be less biodegradable or even

completely refractory when combined with lignin (Jimenez et al., 1990; Tong et al., 1990). Furthermore, Buffiere et al. (2006) showed a link between the methane potential of various lignocellulosic residues and the sum of their cellulose and lignin contents: the higher the sum of cellulose and lignin, the lower the methane potential. Negative correlations were found between the lignin content and biochemical methane potentials for manure and energy crops (R^2=0.88) (Triolo et al., 2011). As pure cellulose can be fully converted into biogas, previous results show that the anaerobic digestion of lignocellulosic biomass is strongly conditioned by the bioaccessibility of cellulose. However, Klimiuk et al. (2010) found that the methane yields of two varieties of miscanthus having comparable lignin concentrations varied by a factor of 2: 100 and 190 L CH_4 kg^{-1} VS for *Miscanthus x giganteus* and *Miscanthus sacchariflorous*, respectively (Klimiuk et al., 2010). Lignin concentration is thus a key parameter in anaerobic biodegradation, though not the only one.

Table I. 3 *Methane potentials from lignocellulosic substrates according literature data*.

Substrate	**Methane yield (L CH_4 kg^{-1} VS_{added})**	**Energy yield (MJ kg^{-1} VS_{added})[a]**	**References**
Newsprint	97	3.86	(Xiao and Clarkson, 1997)
Switchgrass	125	4.97	(Guiot et al., 2009)
Wheat grass	160	6.37	(Romano et al., 2009)
Sisal fibre	180	7.16	(Mshandete et al., 2006)
Wheat staw	182	7.24	(Menardo et al., 2012)
Winter wheat staw	189	7.52	(Amon et al., 2007)
Summer barley straw	189	7.52	(Amon et al., 2007)
Rice straw	190	7.56	(Zhang and Zhang, 1999)
Rice straw	195	7.76	(Dinuccio et al., 2010)
Rice straw	197	7.84	Menardo et al., 2012)
Willow	200	7.96	(Uellendahl et al., 2008)
Miscanthus	200	7.96	(Uellendahl et al., 2008)
Wheat straw	204	8.11	(Sambusiti et al., 2012c)
Sugar beet leaves	210	8.36	(Amon et al., 2007)
Paper tube residues	222	8.83	(Teghammar et al., 2009)
Rice straw	224	8.91	(Ghosh and Bhattacharyya, 1999)
Barley straw	229	9.11	(Dinuccio et al., 2010)
Grass hay	230	9.15	(Lehtomaki et al., 2004)
Bagasse	237	9.43	(Kivaisi and Eliapenda, 1994)
Barley straw	240	9.55	Menardo et al., 2012)
Maize stalks	246	9.78	(Menardo et al., 2012)
Ensiled sorghum forage	269	10.7	(Sambusiti et al., 2012c)

Substrate	Methane yield (L CH_4 kg^{-1} VS_{added})	Energy yield (MJ kg^{-1} VS_{added})[a]	References
Wheat straw	270	10.74	(Jackowiak et al., 2011)
Switchgrass	296	11.77	(Jackowiak et al., 2010)
Wheat straw	297	11.82	(Kaparaju et al., 2009)
Sunflower sillage	300	11.93	(Amon et al., 2007)
Sugar beet tops	310	12.33	(Lehtomaki et al., 2004)
Cynara stalks	310 - 500	12.33	(Oliveira et al., 2010)
Maize	317-321	12.6	(Dinuccio et al., 2010) (Pakarinen et al., 2011)
Potato pulp	332	13.21	(Kryvoruchko et al., 2008)
Winter rye	336	13.37	(Petersson et al., 2007)
Sunflower sillage	345	13.73	(Bauer et al., 2009)
Sorghum sillage	362	14.4	(Bauer et al., 2009)
Office paper	364	14.48	(Xiao and Clarkson, 1997)
Maize sillage	370	14.72	(Bruni et al., 2010)
Barley sillage	375	14.92	(Bauer et al., 2009)
Maize sillage	390	15.51	(Amon et al., 2007)
Summer switchgrass	403	16.04	(Frigon et al., 2008)
Grass sillage	431	17.15	(Pakarinen et al., 2009)

[a] Energy yield $1Nm^3$ CH_4 = 39790 kJ
[b] mL CH_4 kg^{-1} TS added or MJ kg^{-1} TS added

3.3 Coupling biohydrogen and methane in two-stage process

Some studies have been carried out using two-stage H_2/CH_4 process (Table I.4). Kongjan et al., (2010) have investigated two-stage process from wheat straw in continuous thermophilic reactors. Hydrogen and methane production of 89 L H_2 kg^{-1} VS and 307 L CH_4 kg^{-1} VS were evaluated (Kongjan et al., 2010). A two-stage process was also applied to potatoes with hydrogen production of 271 L H_2 kg^{-1}VS and subsequent methane production of 130 L CH_4 kg^{-1} VS (Xie et al., 2007). Hawkes et al., (2008) measured a hydrogen production from wheatfeed between 56 and 64 L H_2 kg^{-1} wheatfeed and a theroretical methane potential around 262 L CH_4 kg^{-1} wheatfeed was evaluated using the outlet of the H_2 dark fermentation process. This kind of process was also studied in continuous reactors by Antonopoulou et al. (2006) using sweet sorghum. The sugar-rich hydrolysate was extracted in top water at 30°C during 1h and separated from the remaining solid fraction. The two-stage H_2/CH_4 process was applied to the hydrolysed part that was rich in readily-fermentable sugars whereas only methane production was considered on the solid

fraction. Yields of 10.4 L H_2 kg^{-1} TS and 29 L CH_4 kg^{-1} TS were achieved for the hydrolysate and 78 L CH_4 kg^{-1} TS for the solid fraction.

However, few studies have investigated the benefit of using two-stage H_2/CH_4 compared to one stage CH_4 process. Pakarinen et al (2009) compared mesophilic CH_4 production from grass sillage in a one-stage process to combined thermophilic H_2 and mesophilic CH_4 production in a two-stage process. As well as a hydrogen production of 5.6 L H_2 kg^{-1} VS, a 8 % increase in CH_4 yields was obtained from grass sillage in the two-stage process (467 L CH_4 kg^{-1} VS) compared to the one-stage process (431 L CH_4 kg^{-1} VS) (Pakarinen et al., 2009). In terms of energy, an increase of 7% in MJ kg^{-1} VS compared to one-stage CH_4 was observed with the two-stage process, in which only 0.4 % came from hydrogen production. This higher methane yield in the two-stage process was attributed to the fact that the thermophilic H_2 production stage enhanced hydrolysis of the solid substrates and resulted in increased solubilization and VFA production (Pakarinen et al., 2009). A two-stage process can increase methane production; however, the increase in the CH_4 yield has to be considered in the light of the investment required in the more complex two-stage process (Pakarinen et al., 2009). Moreover, enhancement of methane production through two-stage processes compared to one-stage process seems also highly dependent on the nature of the substrate used. Indeed, Pakarinen et al., 2011 did not notice on maize any energy gain increase between one and two-stage batch processes. Indeed energy of 3210 (± 230) kWh t^{-1}VS and 3438 (± 63) kWh t^{-1}VS were noticed for respectively one and two-stage processes corresponding to an increase of 7%. Moreover previous results were obtained from batch experiments and thus cannot be extrapoled to large scale continuous reactor (Pakarinen et al., 2011).

However, Luo et al. (2012) have investigated a two-stage H_2/CH_4 in continuous reactors on a mixture of cake, glycerol and stillage of rapeseed (65/3/32w/w/w dry matter). The average yield of hydrogen and methane were 45 L H_2 kg^{-1} VS and 347 L CH_4 kg^{-1} VS, respectively. On the contrary, under the same conditions, the single-stage processfor methane production failed, where final pH of the effluent decreased to below 6 and only hydrogen was detected in reactor headspace (Luo et al., 2012). Besides to produce a mixture of biofuels, this continuous experiment has also shown the feasibility of codigestion of different byproducts in a biorefinery concept.

Table I. 4 *Hydrogen and methane production in two-stage process.*

Substrate	Hydrogen yield (L H_2 kg^{-1} VS_{added})	Methane yield (L CH_4 kg^{-1} VS_{added})	Energy yield (MJ kg^{-1} VS_{added})[a]	Energy gain[d] (%)	References
Grass sillage	5.6	467	18.36	8.4 (ns)	(Pakarinen et al., 2009)
Sweet sorghum	10.4 [b]	107 [b]	4.36 [b]	-	(Antonopoulou et al., 2006)
Maize	5.6	342	13.72	7 (ns)	(Pakarinen et al., 2011)
Potatoes	200.4	130	7.33	-	(Xie et al., 2007)
Water hyacinth	26.8 [b]	229 [b]	9.4 [b]	-	(Chuang et al., 2011)
Cornstalks	37.6 [b]	112 [b,c]	4.86 [b]	-	(Cheng and Liu, 2011b)
Wheat straw	89	307	12.21	-	(Kongjan et al., 2010)
Wheatflour	56-64[b]	262-264 [b]	11-11.2 [b]	-	(Hawkes et al., 2008)
Oil seed cakes	24	385	14	3.7 (ns)	(Luo et al., 2011)
Stillage of rapeseed straw	79	382	14.5	1.4 (ns)	(Luo et al., 2011)
Mixture of cake, glycerol and stillage of rapeseed (65/3/32w/w/w dry matter)	45	347	14.3	-	(Luo et al., 2011)

[a] Energy yield 1Nm^3 CH_4 = 39790 kJ; 1Nm^3 H_2 = 10780 kJ.
[b] mL CH_4 kg^{-1} biomass added or mL H_2 kg^{-1} biomass added or MJ kg^{-1} biomass added.
[c] Methane production was considered only on liquid effluent of H_2 fermentation, solid residue after dark fermentation is not considered
[d] Energy gain compared to one-stage CH_4 process
ns: not significatif

3.4 General remarks

According to literature data, hydrogen potentials range from 3.2 L H_2 kg^{-1} VS (corn stalk) to 106 L H_2 kg^{-1} VS (Jerusalem artichoke tubers) and methane potentials from 97 L CH_4 kg^{-1} VS (Newsprint) to 431 L CH_4 kg^{-1} VS (grass sillage). However, such values are still lower from theoretical hydrogen potential from glucose (250 L H_2 kg^{-1} glucose considering an average production of 2 mol H_2 mol^{-1} glucose with mixed cultures) and methane potentials (475 L CH_4 kg^{-1} VS) that can be expected from lignocellulosic substrates (Frigon and Guiot, 2010). Methane potentials do not generally exceed 60% of theoretical values due to poorly- or non-biodegradable compounds (*eg.* lignin, peptidoglycan...) and polymers that are difficult to dissolve (cellulose, hemicelluloses and proteins) (Frigon and Guiot, 2010). Fermentative conversion of lignocelluloses into biohydrogen and methane is limited by the biological hydrolysis step as suggested by

Pavlosthathis and Giraldogomez (1991). Indeed, some compositional and structural characteristics of the lignocellulosic matrix limit the accessibility of holocelluloses. Thus, pretreatments are required to enhance holocelluloses accessibility and further conversion into biohydrogen and methane.

4. Pretreatment categories: impact on compositional and structural features and fermentative processes (biohydrogen and methane) performances

In the lignocellulosic matrix, several compositional and structural features such as lignin content, crystallinity of cellulose, pectin and hemicelluloses contents, porosity, accessible surface area and acetyl groups can limit the microbial or enzymatic accessibility and thus further conversion of lignocellulosic substrates into biofuels. This sub-chapter focusses on the effect of various pretreatment categories on compositional and structural features changes. Pretreatment used as a tool to enhance hydrogen and/or methane productions from lignocellulosic substrates is also discussed. Generally, pretreatment methods are divided into three different categories: physical, thermo-chemical and biological or various combinations of these (Mosier et al., 2005). For the purposes of classification, steam explosion and liquid hot water are excluded from being considered chemical pretreatments since only water and no extraneous chemicals are added to the biomass (Mosier et al., 2005).

4.1 Physical pretreatment

4.1.1 Impact on compositional and structural features

Physical pretreatment methods include chipping, grinding, milling, high-energy radiation using γ-rays, electron beam, microwaves, steam explosion and liquid hot water.

Chipping, grinding, milling (e.g. two-roll, hammer, colloid and vibro ball) pretreatment leads to a reduction in the particule size, usually to 10-30 mm after chipping and 0.2-2 mm after milling or grinding. Mechanical pretreatment transforms the biomass into a fine powder, thus increasing the surface area of the cellulose and reducing the degree of crystallinity of celluloses as well as decreasing the degree of polymerisation of celluloses and hemicelluloses (Galbe and Zacchi, 2007; Palmowski and Muller, 2000; Taherzadeh and Karimi, 2008). The generation of active surface area and the reduction of material structure imply that areas which were difficult to reach for microorganisms and enzymes become accessible (Dumas

et al., 2010). Gharpuray et al. (1983) investigated the effects of ball milling, fitz milling and roller milling on the structural features (crystallinity, surface area and lignin content) of wheat straw. Ball milling pretreatment was found to be effective in increasing the specific surface area (2.3 m^2 g^{-1} pretreated substrate compared to 0.64 m^2 g^{-1} for raw wheat straw) and decreasing the crystallinity index (23.7 compared to 69.6 for the raw wheat straw). Palmowski and Muller (2003) have also studied the effect of comminution on different organic samples (apples, rice, sunflower seeds, hay and maple leaves). After comminution of these substrates, a release of soluble organic compounds occurred for two reasons: cells were destroyed through comminution; and/or the dissolution of organic components through newly-generated accessible surfaces (Palmowski and Muller, 2003). However, Bridgeman et al. (2007) showed that a big reduction in the size of switchgrass is undesirable as it causes significant carbohydrate losses. Moreover, this process is not cost-effective because it requires too much energy and it has been shown that greater amounts of energy are needed to reduce size when biomass has higher moisture content (Yu et al., 2006).

During **liquid hot water** (LHW) treatment, the lignocellulosic substrate is heated to a high-temperature (200-230°C) for a few minutes. Water under high pressure can penetrate into the biomass, increasing surface area and hence removing hemicelluloses and lignin. Generally, all the hemicelluloses, 35-60% of the lignin and 4-22% of the cellulose are dissolved (Mosier et al., 2005; Wyman et al., 2005). Three types of reactor can be used for liquid hot water pretreatment: co-current (biomass and water are heated together for a certain residence time), counter-current (water and lignocelluloses move in opposite directions), and flow-through (hot water passes over a stationary bed of lignocelluloses) (Liu and Wyman, 2005).

During **steam explosion**, lignocellulosic biomass is heated rapidly to a high temperature (160-260°C) with sufficient pressure (7-50 bar) to enable water molecules to penetrate the substrate structure for a few minutes. The pressure is then suddenly released to allow the water molecules to escape in an explosive manner. This pretreatment opens up the plant cells, increases surface area and enhances the digestibility of biomass (Ballesteros et al., 2000). One limitation of steam explosion is the incomplete disruption of the lignin-carbohydrate matrix (Kumar et al., 2009a). Consequently, steam pretreatment can be improved by using an acid catalyst, such as H_2SO_4 or SO_2 (1-2% w/w), which increases the recovery of hemicelluloses

sugars (Galbe and Zacchi, 2007). SO_2 steam explosion at 190°C for 2 min was applied to wheat straw and spruce: solubilization of hemicelluloses was observed at, respectively, 46 % and 85% (Li and Chen, 2007).

Among mechanical pretreatment, digestibility of lignocellulosic biomass can also be enhanced by use of **high-energy radiation using γ-rays, electron beam or microwaves**. Kumakura and Kaetsu (1983) investigated the effect of irradiation pretreatment on bagasse: after enzymatic hydrolysis, a double yield of glucose was observed for the pretreated sample. Cleavage of β-(1,4)-glycosidic bonds leading to an increase in surface area and a reduction in crystallinity was observed after applying γ-rays to cellulose (Takacs et al., 2000). Microwave pretreatments have been investigated on lignocellulosic substrates as wheat straw and switchgrass (Jackowiak et al., 2011; Jackowiak et al., 2010). Microwave pretreatment enhanced significantly the solubilization of lignocellulosic substrates and microwave pretreatment of switchgrass allowed an augmentation of glucose in supernatant, from a concentration of 400 mg L^{-1} at 90 °C until almost 1 g L^{-1} at 150 °C (Jackowiak et al., 2010). Generally, microwave irradiation can change the ultrastructure of cellulose, degrade hemicelluloses and increase the accessibility of the substrate (Zhu et al., 2005). In most cases, replacing classical heating by microwave irradiation led to spectacular accelerations, due to the efficient internal heating produced by the direct coupling of microwave energy with the molecules present in the reaction mixture as opposed to a slow and inefficient energy transfer when using classical heating (Kappe, 2008). Microwave pretreatment combined with acid pretreatment (HCl) was used on wheat straw and wheat bran (Fan et al., 2005; Pan et al., 2008). The total soluble sugar in the microwave-assisted acid-pretreated wheat bran increased from 0.086 g g^{-1} TS to 0.461 g g^{-1} TS at 9 min hydrolysis time. Monteil-Rivera et al., (2012) investigated microwave (MW)-assisted extraction of lignin from triticale straw. Maximal lignin yield (91 %) was found when using 92 % ethanol, 0.64 N H_2SO_4, at 148 °C (Monteil-Rivera et al., 2012). However microwave pretreatment has several disadvantages, including high energy consumption, complex operation procedures and strict monitoring of equipment (Pan et al., 2008).

4.1.2 Impact on hydrogen or methane production

Studies which have investigated the use of physical pretreatments to enhance **hydrogen production** from lignocellulosic residues are summarized in Table I.5 and Table I.6. Up to now only Yuan et al. (2011) have

investigated the effect of particle size reductions on hydrogen production from wheat stalk. With a solid loading of 2% (w TS /v), the cumulative hydrogen production of particle size increased from 14.5 L H_2 kg-1 VS at particle size of 10 mm to 17.6 L H_2 kg^{-1} VS at particle size of 1 mm (Yuan et al., 2011). Combined acid-microwaves pretreatments was found interesting to enhance significantly hydrogen production of wheat straw as hydrogen production increase from 0.5 L H_2 kg^{-1} VS (raw wheat straw) to 68 L H_2 kg^{-1} VS (Fan et al.,2005). On the contrary, Ozkan et al. (2010) noticed a decrease of 26 % of hydrogen potentials of beet-pulp after alkaline-microwaves pretreatments. Steam explosion has been also investigated for hydrogen production with corn straw, cornstalks and wheat bran, giving hydrogen production of 68 mL H_2 g^{-1} TS, 63.7 mL H_2 g^{-1} TS and 86 mL H_2 g^{-1} VS, respectively (Li and Chen, 2007; Lu et al., 2009; Pan et al., 2008). In the case of wheat bran, an energy gain of 69 % compared to raw substrate was noticed (Pan et al., 2008).

As for **methane production**, mechanical pretreatments, in particular grinding, have been used to enhance anaerobic digestion. Grinding (0.5mm) led to a 53% enhancement of the energy yield from wheat straw (Sharma et al., 1988). The influence of particle size reduction on different types of organic solid waste was investigated by Palmowski and Muller (2000). It was observed that such size reduction of a substrate led to an increase in methane production (≈ 20%) and a reduction in anaerobic digestion time (≈ 20%), particularly with substrates having a high fibers content such as hay and leaves (Palmowski and Muller, 2000). According to Palmowski and Muller (2000), comminution not only releases cell compounds usable more easily and rapidly but also supports hydrolysis of the solid compounds in the long term. Mshandete et al. (2006) studied the degradation and biogas potential of sisal fiber with sizes ranging from 2 to 100 mm. It was shown that the methane yield was inversely proportional to particle size with an increase of 22% when the fibers were cut at 2 mm size (220 L CH_4 kg^{-1} VS for 2mm, compared to 180 L CH_4 kg^{-1} VS for untreated fibers). Recently, Menardo et al., (2012) found an increase of methane potentials after mechanical pretreatment of barley straw (by 54 % for particle size of 0.5 cm) and wheat straw (by 83.5 % for particle size of 0.2 cm). On the contrary, no significant methane potentials improvement was noticed for rice straws and maize stalks. Similarly, Dumas et al., (2010) did not find significant differences on the methane potentials of wheat straw after a cutting milling and centrifugal grinding between 804 and 45 µm particle sizes. Similar trends have been observed by Sambusiti et al., (2012a) who did not observe any difference in

methane potential of ensiled sorghum forage at four particles sizes of 721, 577, 269 and 162 µm. Among mechanical pretreatments, microwaves at 150°C pretreatments was found efficient to enhance methane potentials of 8 % and 28 % respectively for switchgrass and wheat straw (Jackowiak et al., 2010, 2011). On the contrary Li et al. (2012) have shown that microwave pretreatment on energy grass led to decreases up to 18 % and 12 % for methane production rate and methane potential respectively (Li et al., 2012). Then, ultrasound pretreatments were found efficient to enhance methane potentials from sunflower oil cakes (+54%) and corn ethanol by-products (+41%) (Fernandez-Cegri et al., 2012; Wu-Hann, 2008).

Steam explosion has been also widely investigated. When steam explosion at 180°C for 25 min was applied to wheat straw, methane production increased by 31% (Bauer et al., 2009). Teghammar et al. (2009) combined steam explosion with chemical pretreatment. They observed that the combination of steam explosion with 2% NaOH and 2% H_2O_2 enhanced the methane yield of paper tube residues from 238 L CH_4 kg^{-1} VS to 493 L CH_4 kg^{-1} VS (Teghammar et al., 2009). Liquid Hot Water pretreatments have been widely investigated to enhance methane production from lignocellulosic residues (Menardo et al., 2012; Sambusiti et al., 2012b). Sambusiti et al. (2012b) have noticed a 10 % increase of methane potentials of wheat straw thermally pretreated at 160°C during 1h. After thermal pretreatment at 120°C during 1h, Menardo et al. (2012) showed 32 % and 64 % methane increase respectively for rice straw and wheat straw (Menardo et al., 2012).

Table I. 5 *Biohydrogen or methane potentials enhancement from physically pretreated (grindind, ultrasound or microwaves) lignocellulosic residues.*

Categories	Lignocellulosic material	Pretreatment conditions	$BioH_2$ production (L H_2 kg^{-1} VS_{added})	CH_4 production (L CH_4 kg^{-1} VS_{added})	Energy from pretreated biomass [a] (MJ kg^{-1} VS_{added})	Energy from raw biomass [a] (MJ kg^{-1} VS_{added})	Energy gain (%)	References
Mechanical	Wheat stalk	Grinding, 1 mm	17.6		0.19		-	(Yuan et al., 2011)
	Wheat straw	Grinding, 0.5 mm		248	9.87	6.45	53	(Sharma et al., 1988)
	Bermuda grass	Grinding, 0.4 mm		228	9.07	5.45	66	(Sharma et al., 1988)
	Cynara stalks	Milling and sieving, 2 mm		-	-	-	1.5	(Oliveira et al., 2010)
	Cynara stalks	Milling and sieving, 0.4 mm		-	-	-	36	(Oliveira et al., 2010)
	Maize sillage	Milling, 2-8mm		410	16.31	14.72	11	(Bruni et al., 2010)
	Sisal fibre	Milling, 2 mm		220	8.75	7.16	22	(Mshandete et al., 2006)
	Wheat straw	Cutting mill and centrifugal grinding, 0.804-0.045 mm		302	12	12	0	(Dumas et al., 2010)
	Barley straw	Knife mill, 5 mm		370	14.72	9.54	54	(Menardo et al., 2012)
	Wheat straw	Knife mill, 2 mm		334	13.28	7.24	83.5	(Menardo et al., 2012)
Ultrasound	Sunflower oil cakes	Ultrasounds (16.6 min, 24000 kj . kg^{-1}TS)		275	10.93	7.08	54	(Fernandez-Cegri et al., 2012)
	Corn ethanol by-products	Ultrasounds (50s, amplitude: 100%)		459	18.26	12.53	41	(Wu-Hann,2008)
Microwaves	Beet-pulp	Alkaline (pH=12, 30min) + Microwaves (170°C, 700W, 30 min)	86.5		3.4	4.65	-27	(Ozkan et al., 2010)
	Wheat straw	Acid (2% HCl) + microwaves (heating time:8min)	68.1		0.73	0.005	1460	(Fan et al., 2005)
	Wheat bran	0.01M HCl + 9 min microwave (800 W)	93		1	0.55	81	(Pan et al., 2008)
	Switchgrass	Microwaves 2450MHz, 150°C		320	12.7	11.8	8	(Jackowiak et al., 2010)
	Wheat straw	Microwaves 2450MHz, 150°C (ramp time: 5°C/min)		345	13.7	10.74	28	(Jackowiak et al., 2011)

[a] Energy yield 1Nm^3 CH_4 = 39790 kJ; 1Nm^3 H_2 = 10780 kJ

Table I. 6 *Biohydrogen or methane potentials enhancement from physically pretreated (steam explosion and liquid hot water) lignocellulosic residues.*

Categories	Lignocellulosic material	Pretreatment conditions	$BioH_2$ production (L H_2 kg^{-1} VS_{added})	CH_4 production (L CH_4 kg^{-1} VS_{added})	Energy from pretreated biomass [a] (MJ kg^{-1} VS_{added})	Energy from raw biomass [a] (MJ kg^{-1} VS_{added})	Energy gain (%)	References
Steam explosion	Corn straw	1.5 MPa, 10 min, + cellulase (25 FPU / g)	68[b]		0.73[b]	-	-	(Li and Chen, 2007)
	Wheat bran	0.27 MPa, 60 min, 0.01M HCl	86		0.93	0.55	69	(Pan et al., 2008)
	Japanese cedar chips	4.51MPa / 258°C		365	14.52	0	-	(Take et al., 2006)
	Bamboo	5min / 243°C		215	8.55	-	-	(Kobayashi et al., 2004)
	Potato pulp	15min / 107°C		373	14.84	13.21	12	(Kryvoruchko et al., 2008)
	Wheat straw	10 min / 170°C		361	14.36	10.98	31	(Bauer et al., 2009)
	Paper tube residues	10min / 220°C / 4% H_2O_2 (w/w) + 4% NaOH (w/w)		493	19.62	8.83	122	(Teghammar et al., 2009)
	Cornstalks	5 min / 1.6 MPa	7.8[c]	13.1[c]		-	-	(Lu et al., 2009)
	Japanese cedar chips	170 °C / 30 min		28	1.11	0	-	(Take et al., 2006)
Hot water	Wheat straw	120°C/ 1h		299	11.89	7.25	64	(Menardo et al., 2012)
	Rice straw	120°C/ 1h		261	10.38	7.86	32	(Menardo et al., 2012)
	Barley straw	90°C/ 1h		340	13.52	9.52	42	(Menardo et al., 2012)
	Maize stalks	120°C/ 1h		267	10.62	9.74	9	(Menardo et al., 2012)
	Sorghum sudanense	100°C/ 1h		282	11.22	10.68	5	(Sambusiti et al., 2012b)
	Wheat straw	160°C/ 1h		224	8.91	8.1	10	(Sambusiti et al., 2012b)
	Cynara stalks	160°C/ 1h		620	24.67	19.9	24	(Oliveira et al., 2012)

[a] Energy yield $1Nm^3$ CH_4 = 39790 kJ; $1Nm^3$ H_2 = 10780 kJ.
[b] mL CH_4 kg^{-1} TS added or MJ kg^{-1} TS added
[c] mL CH_4 kg^{-1} biomass added or mL H2 kg^{-1} biomass added or MJ kg^{-1} biomass added

4.2 Thermo-chemical pretreatment

4.2.1 Impact on compositional and structural features

Acid pretreatment is used for efficiently removing hemicelluloses by breaking ether bonds in lignin/phenolics-carbohydrates complexes without dissolving lignin (Knappert et al., 1981). Among acidic pretreatments, sulfuric acid is the most widely used but other acids have been used, including hydrochloric, phosphoric, maleic, acid or nitric acids (Fernandes et al., 2009; Taherzadeh and Karimi, 2008).

Concentrated acids (typically 72% H_2SO_4 or 42% HCl at low temperature) usually lead to the conversion of at least 90% of the potential glucan in the biomass into glucose (Xiao and Clarkson, 1997). However, concentrated acids are corrosive and toxic and need to be recovered after pretreatment to make the process economically feasible (Sun and Cheng, 2002). Consequently, dilute acid pretreatment appears to be a more promising process and has been widely studied. Concentrations of 0.4-2% H_2SO_4 at temperatures between 160-220°C for a few minutes are typically employed (Willfor et al., 2005). Dilute sulfuric acid treatment has been used successfully to hydrolyze hemicelluloses to sugars with high yields, to change the structure of the lignin and to increase the cellulosic surface area (Mosier et al., 2005; Wyman et al., 2005). Peracetic acid, which is also an oxidant (oxidation potential: 1.81 eV) was shown to lead to a drastic reduction in the crystallinity index; this was attributed to structural swelling and dissolution of the crystalline cellulose component (Gharpuray et al., 1983). The disadvantages of acid pretreatment are the use of a corrosive reagent, with corresponding downstream neutralization, and special materials for reactor construction.

Alkaline pretreatment involves the use of bases such as sodium, potassium, calcium and ammonium hydroxide**.** Alkaline pretretament is used mainly for the cleavage of ester bonds in lignin/phenolics-carbohydrates complexes (Buranov and Mazza, 2008). It leads to the saponification of the uronic bonds between hemicelluloses and lignin, swells the fibers and increases pore size, facilitating the diffusion of the hydrolytic enzymes (Datta, 1981). Aqueous lime or soda pretreatment was shown to be effective at a lower temperature than that used in acid treatment but the time required is of the order of hours or days rather than the minutes or seconds needed for acid pretreatment. For example, the solubilization of lignin (14%) and the increase in the accessibility of holocelluloses were observed after $Ca(OH)_2$ pretreatment was applied on wheat straw at 85°C for 3 h (Chang et al., 1998). Pretreatment of miscanthus with 12% NaOH at

70°C for 4 h led to 77% of delignification of the raw material and to 44 % of hydrolysis of hemicelluloses (de Vrije et al., 2002). Gupta and Lee, (2010b) have shown that sodium hydroxide pretreatment supplemented with hydrogen peroxide significantly raises delignification of two different species of hybrid poplars: high lignin and low lignin poplar.

Aqueous ammonia can also be used as alkaline pretreatment to enhance the enzymatic accessibility (Gupta and Lee, 2009b). Pretreatment based on aqueous ammonia was investigated on two substrates, one with low lignin content (corn stover) and the other one with high lignin content (hybrid poplar) (Gupta et al., 2007). Aqueous ammonia has been proven effective in treatment of low lignin feedstocks as corn stover but less for high lignin feedstock as hybrid poplar (Gupta et al., 2007). Ammonia can also be used in the ammonia recycle percolation (ARP) pretreatment. Aqueous ammonia passes through the biomass in a percolation reactor (packed-bed, flow-through type) at high temperatures (150-170°C). Under these conditions, ammonia reacts with lignin and not with cellulose. ARP is effective for the delignification of hardwood and agricultural residues but less effective for softwood (Mosier et al., 2005). For example, in corn stover, ARP removed 75-85 % of the total lignin and solubilized 50-60 % of hemicelluloses, but retained more than 92 % of the cellulose content (Kim and Lee, 2005). In the case of herbaceous biomass, Iyer et al (1996) observed 65-85% delignification of switchgrass.

Ammonia fiber explosion (AFEX) is a physicochemical pretreatment using also liquid ammonia in which the biomass is exposed to at a relatively high temperature (90-120°C) for a period of 30 min, followed by the sudden reduction of pressure. AFEX pretreatment reduces lignin content, increases surface area, and cellulose and hemicelluloses are well preserved, showing little or no degradation (Moniruzzaman et al., 1997). AFEX was shown to be insufficiently effective for substrates with high lignin content such as aspen in the form of chips or wood (McMillan, 1994). For example, Gupta and Lee (2010a) applied AFEX to switchgrass and observed 68%, 45% and 1% of solubilization of lignin, hemicelluloses and cellulose, respectively. On the other hand, Kumar and Wyman (2009b) observed no solubilization (neither of lignin, hemicelluloses nor cellulose) after treatment of poplar. However, the crystallinity index of corn stover was significantly reduced (from 50.3 to 36.3) whereas the crystallinity index of poplar did not change (Kumar and Wyman, 2009b).

Oxidative pretreatment (H_2O_2, O_3) can also be used to solubilize lignin and hemicelluloses and to increase the surface area of cellulose. Hydrogen peroxide is usually used in association with alkali (pH=11.5) (Rabelo et al., 2008). For example, 50 % lignin and most of the hemicelluloses were solubilized by 2 % H_2O_2 at pH=11.5 and 30°C for 8 h on sugarcane bagasse (Azzam, 1989). By applying 1% H_2O_2 at pH=11.5 and 65°C for 3h on corn stover, 66 % delignification was observed compared to untreated corn stover (Selig et al., 2009). Ozone can be applied to degrade lignin, though hemicelluloses is slightly affected and cellulose not at all (Kumar et al., 2009a). For example, a reduction in lignin content from 29% to 8% was observed after ozonolysis pretreatment of poplar sawdust (Vidal and Molinier, 1988). Ben-Ghedalia and Miron (1981) have shown 60 % removal of lignin by applying ozone pretreatment to wheat straw. Although the process is carried out at room temperature and normal pressure, this pretreatment requires a large amount of ozone, making the process expensive.

Inorganic salts (NaCl, KCl, $FeCl_3$...) have also been tested as catalysts for the degradation of hemicelluloses in corn stover. $FeCl_3$ pretreatment was the most effective on corn stover, removing almost all the hemicelluloses; this pretreatment can disrupt almost all the ether linkages and some ester linkages between lignin and carbohydrates but had no effect on delignification. $FeCl_3$ significantly increased the degradation of hemicelluloses in aqueous solutions heated to between 140°C-200°C, with 90% xylose solubilization and only 10 % cellulose removal (Liu et al., 2009a; Liu et al., 2009b).

Ionic liquids also commonly called "green solvents" have also been investigated as an aid to dissolve lignocellulosic biomass (Dadi et al., 2007; Nguyen et al., 2010; Samayam and Schall, 2010). Ionic liquids offer several advantages: they have minimal environmental impact due to their low-volatility and can be reused after pretreatment (Dadi et al., 2007). Indeed, the low vapour pressure of ionic liquids make them more than 99 % recoverable in a number of operations, thus reducing costs of solvents usage (Brodeur et al., 2011). Ionic liquid pretreatment of lignocellulosic biomass produces amorphous cellulose with little residual crystallinity (Samayam and Schall, 2010). Poplar and switchgrass were pretreated with (Emin) OAc (1-ethyl 3-methyl imidazolium acetate) for 30 min at 120°C. For poplar and switchgrass, the crystallinity dropped from 38% to 8 % (wt %) and from 21% to 6%, respectively (Samayam and Schall, 2010). Besides reducing crystallinity, ionic liquids can efficiently remove lignin. Wood floor was pretreated with (Emin)OAc for 90 min at temperatures ranging from 50 to 130°C. At 110°C, 44% of the

lignin was removed and the crystallinity reduced from 63 % to 30% (Lee et al., 2009). At the present time, however, this process is too expensive for application as a lignocellulosic pretreatment due, mainly, to the high cost of ionic liquids (Nguyen et al., 2010).

In the **organosolv process**, an organic or aqueous organic solvent mixture with inorganic acid catalysts is used to break the internal lignin and hemicelluloses bonds. The solvents generally used are methanol, ethanol, acetone, ethylene glycol, triethylene glycol, tetrahydrofurfuryl alcohol; the acids used are HCl or H_2SO_4 (Kumar et al., 2009a). For most organosolv processes at high temperatures (185-210°C), there is no need for the addition of acid because organic acids released from the biomass at this temperature act as catalysts for the breakdown of the lignin-carbohydrate complex (Duff and Murray, 1996). Most hemicelluloses and lignin are solubilized, but the cellulose remains solid, making this process competitive for the bioethanol process (Zhao et al., 2009). During the organosolv process with poplar using aqueous ethanol; the recovery of lignin and hemicelluloses were 74 % and 72 %, respectively (Pan et al., 2006). In the case of an ethylene glycol pretreatment of wheat straw, solubilization was observed at a level of 95 % for hemicelluloses and 64 % for lignin (Gharpuray et al., 1983). However, at the end of organosolv pretreatment, the solvents used in the process must be removed from the reactor because they may inhibit the growth of micro-organisms and they need to be recycled in order to reduce costs (Kumar et al., 2009a).

In **CO_2 explosion**, the biomass is exposed to CO_2 at low temperatures (30-50°C) and high pressure (140-180 bar) for a short period of time (min or hours), followed by a sudden drop in pressure. CO_2 explosion is similar to steam explosion and AFEX: carbon dioxide molecules are comparable in size to those of water and ammonia and are able to penetrate small pores accessible to water and ammonia molecules. With the explosive release of carbon dioxide pressure, disruption of the cellulosic structure increases the accessible surface area (Zheng et al., 1998). Moreover, an increase in pressure facilitates the faster penetration of carbon dioxide molecules into the crystalline structures and more glucose is produced in further biological hydrolyse of biomass. Once dissolved in water, carbon dioxide forms carbonic acid. Even though it is a weak acid, it should be helpful in hydrolysing hemicelluloses as well as cellulose (Zheng et al., 1998). CO_2 explosion is more cost-effective than steam explosion because the temperature required in the process is lower. It was also shown to be more cost-effective than ammonia explosion (Zheng et al., 1998). A further advantage of using CO_2 explosion and AFEX rather than steam explosion is that they both avoid xylose

decomposition which produces furfural, an inhibitor of the biological process involved in bioethanol production (Dale and Moreira, 1982).

Wet oxidation pretreatment involves the treatment of the biomass with air or oxygen at temperatures above 120°C for few minutes. It was presented in the early 1980s as an alternative to steam explosion. This process is an effective method for separating the cellulosic fraction from lignin and hemicelluloses. The hemicelluloses are cleaved to monomeric sugars, lignins undergo both cleavage and oxidation and cellulose is partly degraded (Saha, 2003; Schultz et al., 1984). Wet oxidation at 195°C for 15 min led to the solubilization of 95 % of hemicelluloses and 40-50% of lignin of sugarcane bagasse (Martin et al., 2007). On the other hand, the combination of wet oxidation with alkaline pretreatment permits the reduction of temperature in the process and, consequently, avoided the formation of soluble compounds such as furfural (Ahring et al., 1996; Martin et al., 2007).

4.2.2 Impact on hydrogen or methane production

Thermo-chemical pretreatments have also been studied with the aim to enhance **biohydrogen** production (Table I.7 and Table I.8). Dilute acid pretreatment has been most widely investigated in relation to biohydrogen production. The main reason for using dilute acid treatment in hydrogen production is the effect of such pretreatment on the lignocellulosic structure: hydrolysis of hemicelluloses to sugars with high yields, a change in the structure of the lignin, an increase in the cellulosic surface area (Mosier et al., 2005; Wyman et al., 2005). This enhancement of hydrogen production remains very slow or inhibited when hemicelluloses and cellulose are not transformed into monomer sugars. Great increases in hydrogen production, varying from 58 % (wheat bran) to 5300 % (cornstalk) was observed for lignocellulosic substrates subjected to dilute acid (HCl) pretreatment (Pan et al., 2008; Zhang et al., 2006). Han et al., (2012) have made an interesting comparison between alkaline (NaOH), acid (HCl) and oxidative (H_2O_2) pretreatments to enhance hydrogen production from soybean straw. For each chemical reagent various concentrations were tested and best enhancement of hydrogen production of 10.5 L H_2 kg^{-1} VS, 23 L H_2 kg^{-1} VS, 48 L H_2 kg^{-1} VS were respectively observed for NaOH at 0.5 %, H_2O_2 at 16 % and HCl at 4% (Han et al., 2012). Interestingly, they showed that combined alkaline with oxidative pretreatments (0.5 % NaOH, 16% H_2O_2) led to an increase of hydrogen potentials (41 L H_2 kg^{-1} VS) compared to alkaline or

oxidative alone at the same concentration (Han et al., 2012). Considering each chemical reagent alone, dilute acid pretreatment was found to be the best to enhance hydrogen production maybe due to high solubilisation of hemicelluloses during such pretreatments. Similar trend was observed by Cui and Shen et al. (2011) by applying acid (4% HCl (w/v), boiled 30 min) and alkaline pretreament (4% HCl (w/v), boiled 30 min) on grass. Indeed, hydrogen production of 39.5 L H_2 kg^{-1} TS and 72 L H_2 kg^{-1} TS respectively for alkaline and acid pretreatments compared to 4.4 L H_2 kg^{-1} TS for the raw substrate (Cui and Shen et al., 2011)

Thermo-chemical pretreatments have also been investigated to enhance **methane** production (Table I.6 and Table I.7). The application of a NaOH pretreatment to coconut fibers and corn stover led to high methane production increases of 50% and 75%, respectively (Kivaisi and Eliapenda, 1994; Zheng et al., 2009). Best enhancement of 792 % of methane potentials was observed by Neves et al. (2006) after alkaline pretreatment of barley waste, but this pretreatment was performed at high sodium hydroxide concentration of 30 % (w/w) during overnight at 25°C. Zhong et al. (2010) have compared two alkaline pretreatments (sodium hydroxide (8% w/w) and ammonia (5% w/w) on corn stover during 20 days at room temperature. Enhancement of methane potentials of 51 % and 207 % were respectively observed for sodium hydroxide and ammonia pretreatments (Zhong et al., 2010).

Oxidative pretreatments have been poorly investigated to enhance hydrogen and methane production from lignocellulosic residues. Teghammar et al., 2009, reported a low increase of 5% in term of methane potentials after oxidative pretreatments (4% H_2O_2 (w/w) / 190°C / 30min) of paper tube residuals (Teghammar et al., 2009). On the contrary, promising results have been observed using ionic liquids N-methylmorpholine-N-oxide (NMMO) to enhance methane production of lignocellulosic substrate (Teghammar et al., 2011). Indeed, an increase of 608 % of the methane potential from rice straw was observed after ionic liquid (NMMO) pretreatments at 130 °C during 1 hour (Teghammar et al., 2011). To finish, wet oxidation gave interesting results with energy gains of 80 % on miscanthus and willow (Uellendahl et al., 2008).

Table I. 7 *Biohydrogen or methane potentials enhancement from chemically (oxidative and alkaline) pretreated lignocellulosic residues.*

Categories	Lignocellulosic material	Pretreatment conditions	$BioH_2$ production (L H_2 kg^{-1} VS_{added})	CH_4 production (L CH_4 kg^{-1} VS_{added})	Energy from pretreated biomass [a] (MJ kg^{-1} VS_{added})	Energy from raw biomass [a] (MJ kg^{-1} VS_{added})	Energy gain (%)	References
Oxidative	Soybean straw	16 % H_2O_2 (w/v), boiled 30 min	23		0.25	0.06	320	(Han et al., 2012)
	Wheat flour	2 % H_2O_2 (w/v), 4h, 60°C	31		0.34[d]	-	-	(Hawkes et al., 2008)
	Paper tube residues	4% H_2O_2 (w/w) / 190°C / 30min		233	9.27	8.83	5	(Teghammar et al., 2009)
Alkaline	Cornstalks	0.5 % NaOH	57		0.62	0.03	1966	(Zhang et al., 2006)
	Sweet sorghum stalk	0.4% NaOH, 20°C, 24 h	127		1.37	0.56	144	(Shi et al.2010)
	Grass	4 % NaOH (w/v), boiled 30 min	39.5[b]		0.43[b]	0.05[b]	760[b]	(Cui and Shen, 2011)
	Beet-pulp	Alkaline (pH=12, 30min)	150		5.96	0.84	29	(Ozkan et al., 2010)
	Grass sillage	4% NaOH (w/w) / 20°C / 24 h	6.5	473	18.89	18.36	2.8	(Pakarinen et al., 2009)
	Soybean straw	0.5% NaOH (w/v), boiled 30 min	10.5		0.11	0.06	92	(Han et al., 2012)
	Paper tube residues	4% NaOH (w/w) / 190°C / 30min		269	10.7	8.83	21	(Teghammar et al., 2009)
	Grass hay	4% NaOH (w/w) / 25°C / 24 h		270	10.74	9.15	17	(Lehtomaki et al., 2004)
	Sugar beet tops	2% NaOH (w/w) / 20°C / 24 h		350	13.93	12.33	13	(Lehtomaki et al., 2004)
	Corn stover	2 % NaOH (w/w) / 20°C / 3 days		233	9.27	5.29	75	(Zheng et al., 2010)
	Wheat straw	10% NaOH / 40°C / 24 h		291	11.58	7.87	47	(Sambusiti et al., 2012b)
	Ensiled sorghum forage	10% NaOH /40°C / 24 h		346	13.78	10.52	31	(Sambusiti et al., 2012b)
	Corn stover	5% NaOH /20°C / 24 h		372	14.8	10.57	40	(Zhu et al., 2010a)
	Cynara stalks	1.4 % NaOH (w/v) / 160 °C, 20 mins		620	24.67	12.34	90	(Oliveira et al., 2012)
	Grass sillage	7.5% NaOH/100°C/48h		452	17.98	12.93	39	(Xie et al., 2012)
	Corn straw	8 % NaOH (w/w) / 15°C / 20 days		472	18.78	6.12	207	(Zhong et al., 2010)
	Rice straw	5% NaOH / 200°C / 10 mins		133	5.29	2.38	122	(Chandra et al., 2012)
	Barley waste	30 % NaOH (w/w) / 25°C / overnight		222	8.83	0.99	792	(Neves et al., 2006)
	Grass hay	3% $Ca(OH)_2$ (w/w) + 4% Na_2CO_3 (w/w) / 25°C / 72h		270	10.74	9.15	17	(Lehtomaki et al., 2004)
	Rice straw	2% NH_3 / 90°C / 10mm		245	9.75	7.56	29	(Zhang and Zhang, 1999)
	Corn straw	5% NH_3 (w/w) / 15°C / 20 days		316	12.57	6.12	51.3	(Zhong et al., 2010)

[a] Energy yield 1Nm^3 CH_4 = 39790 kJ; 1Nm^3 H_2 = 10780 kJ

[b] mL H_2 kg^{-1} TS added or MJ kg^{-1} TS added.

Table I. 8 *Biohydrogen or methane potentials enhancement from chemically (acid, ionic liquid and wet oxidation) pretreated lignocellulosic residues.*

Categories	Lignocellulosic material	Pretreatment conditions	$BioH_2$ production (L H_2 kg^{-1} VS_{added})	CH_4 production (L CH_4 kg^{-1} VS_{added})	Energy from pretreated biomass [a] (MJ kg^{-1} VS_{added})	Energy from raw biomass [a] (MJ kg^{-1} VS_{added})	Energy gain (%)	References
Acid	Cornstalk	0.2 % HCl , boiled 30 min	150		1.62	0.03	5300	(Zhang et al., 2006)
	Beer lees	2 % (w/v) HCl	53[b]		0.57[b]	0.03[b]	1800	(Cui et al., 2009)
	Poplar leaves	4 % (w/v) HCl	33.5[b]		0.36[b]	0.16[b]	125	(Cui et al., 2010)
	Soybean straw	4% HCl (w/v), boiled 30 min	47.6		0.51	0.06	750	(Han et al., 2012)
	Grass	4 % (w/v) HCl, boiled 30 min	72.2		0.77	0.05	1538	(Cui and Shen, 2011)
	Wheat bran	0.01M HCl, boiled 30 min	81		0.87	0.55	58	(Pan et al., 2008)
	Corncorb	1% HCl / 100°C / 30 min	108		1.16	0.14	728	(Pan et al., 2009)
	Wheat straw	2 % H_2SO_4 / 120°C / 90 min	37.1		0.4	0.06	566	(Nasirian et al., 2011)
	Newsprint	30% acetic acid / 2% HNO_3		271	10.78	3.86	179	(Xiao and Clarkson, 1997)
	Sugarcane bagasse	2 % H_2SO_4 (w/v) / 121°C / 15 min		173	6.88	2.58	166	(Badshah et al., 2012)
	Sunflower oil cakes	1 % HCl (w/w cake) / 170°C / 5 min		289	11,49	7.75	48	(Monlau et al., 2012c)
	Bagasse	1M HCl / 25°C / 30 days		-			32	(Kivaisi and Eliapenda, 1994)
	Coconut fibers	1M HCl / 25°C / 30 days		-			76	(Kivaisi and Eliapenda, 1994)
Ionic liquids	Rice straw	N-methylmorpholine-N-oxide (NMMO) / 130°C / 1h		212	8.43	1.19	608	(Teghammar et al., 2011)
	Triticale straw	N-methylmorpholine-N-oxide (NMMO) / 130°C / 15h		233	9.27	1.35	586	(Teghammar et al., 2011)
Wet oxidation	Miscanthus	-		360	14.32	7.96	80	(Uellendahl et al., 2008)
	Willow	-		360	14.32	7.96	80	(Uellendahl et al., 2008)
	Winter rye	2g/L Na_2CO_3 / 195°C / 15 min / 12 bar O_2		447	17.79	13.37	33	(Petersson et al., 2007)

[a] Energy yield 1Nm^3 CH_4 = 39790 kJ; 1Nm^3 H_2 = 10780 kJ

[b] mL H_2 kg^{-1} TS added or MJ kg^{-1} TS added.

4.3 Biological pretreatment

4.3.1 Impact on compositional and structural features

Biological pretreatments include pretreatments with enzymes or microorganisms (mainly fungi or lactic bacteria for ensiling). Industrial **enzymes** such as cellulases (endoglucanase, exoglucanase and β-glucosidase), xylanases or lignolytic enzymes (laccase, lignin and manganese peroxidase) can be used to breakdown all components of lignocelluloses, including lignin, the polymer most refractory to microbial attack (Lopez et al., 2007). Moreover, the use of pectinase as polygalacturonase and pectate-lyase enzymes can increase the hydrolysis of cellulose (Berlin et al., 2007; Frigon et al., 2012). However, the high cost of these industrial enzymes is a limitation for further application at industrial scale.

To overcome the high cost of industrial enzymes, use of **microorganisms such as fungi** (brown-, white-, and soft-rot fungi) that secrete extracellular enzymes can be used (Galbe and Zacchi, 2007). Indeed, such enzymes are abundantly found in forest leaf litter/composts and especially in fungi. These microorganisms possess enzyme systems to attack, depolymerize and degrade the polymers in lignocellulosic substrates (Saritha et al., 2012). Brown-rot fungi mainly attack cellulose, whereas white and soft-rot fungi attack both cellulose and lignin (Galbe and Zacchi, 2007). Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase (Lee et al., 2007).

For example, white-rot fungi (*Ceriporiopsis subvermispora* and *Cyathus stercoreus*) were found effective in delignification of bermuda grass: after incubation with *Ceriporiopsis subvermispora* and *Cyathus stercoreus*, about 23 % and 41 % of total aromatics were removed respectively (Akin et al., 1995). Lee et al. (2007) have investigated the effect of three white-rot fungi (*Ceriporia Lacerata, Stereum hirsutum, Polyporus brumalis)* on Japanese red pine. Among the three white-rot fungi, *Stereum hirsutum* selectively degraded the lignin of the wood sample rather than holocelluloses. Indeed loss of 14.5 % and 7.8 % were repectively noticed for lignin and holocelluloses (Lee et al., 2007). Lopez et al. (2007) used *Coniochaet ligniaria* fungus to pretreat pepper plant residues. This microorganism produced cellulase, xylanase and two lignolytic enzymes (manganese peroxidase and lignin peroxidase). After 20 days of culture, reductions of about 40 %, 50 % and 75 % were obtained for lignin, cellulose and hemicelluloses respectively.

Preteating corn straw by fungi *Pleurotus Florida* during 60 days led to 18.3 %, 36 % and 45 % removals respectively for cellulose, hemicelluloses and lignin (Zhong et al., 2011).

Ensiling can also be considered as a biological natural pretreatment (Neureiter et al., 2005). Various studies have considered the anaerobic digestion of biomass silage. Ensiling is a preferential method to maintain the energy content of the crops, ensuring a good nutritional value when used as feed (Vervaeren et al., 2010). The main objective of ensiling is to induce anaerobic conditions in which the lactic bacteria, which is present in plant can convert mainly water soluble carbohydrates into organic acids. Formation of lactic acid during ensiling is the main acidifying agent preserving structural carbohydrates and proteins (Pakarinen, 2012a). Additives such as acids and bases are currently used to accelerate the pH change to prevent the growth of unwanted microorganisms and further limit the loss of carbohydrates or formation of other acids (Pakarinen, 2012a). Indeed, excessive formation of acids can lead to further decrease of pH in the digester and thus inhibition of methanogens. Lehtomaki (2006) has shown that ensiling has a positive impact on methane production and suggested that the structural polysaccharides contained in plant material, which are quite resistant to anaerobic degradation, can be partially degraded by lactic bacteria during storage (Lehtomaki, 2006).

The various types of biological pretreatment are considered environmentally friendly and energy-saving as they are performed at low temperature and do not need any chemicals but they present very low treatment rates. Moreover, in the case of pretreatments using enzymes or fungi, aseptic conditions or free oxygen area are needed to avoid the consumption of free sugars by indigenous microorganisms present initially on the lignocellulosic substrates (Quemeneur et al., 2012a). In this case, direct addition of enzymes in the fermentative process could be recommended for concomitant release and degradation of the monomeric sugars units (Romano et al., 2009; Quemeneur et al., 2012a).

4.3.2 Impact on hydrogen or methane production

Biohydrogen can be enhanced by using biological pretreatment involving micro-organisms (e.g. brown-, white- and soft-rot fungi, aerobic bacteria) or enzymes (cellulases, xylanases, lignin peroxidase, manganese peroxidise and pectinases) (Ghosh and Bhattacharyya, 1999; Lehtomaki et al., 2004; Frigon et al., 2012) as shown in table I.9 and table I.10. Cui et al., (2010) noticed an increase of the hydrogen yield of poplar

leaves from 15.04 to 44.92 mL H_2 g^{-1}-dry poplar leaves by pretreatment with 2% Vicozyme L (a mixture of arabinase, cellulase, β-glucanase, hemicellulase and xylanase). A two fold increase of hydrogen potentials was observed after enzymatic pretreatments (mixture of cellulase and hemicellulases) of wheat straw (Quemeneur et al., 2012a). Aerobic bacterium *Bacillus amyloliquefaciens* increased the energy from 0.18 (untreated maize) to 0.78 MJ kg^{-1} VS corresponding to an increase of 333% (Ivanova et al., 2009).

Finally, some studies have investigated combined chemical-enzymatic pretreatment to enhance biohydrogen production from lignocellulosic substrates. Indeed, Chairattanamanokorn et al. (2009) investigated a combination of alkaline follow by enzymatic pretreatment. By applying 4% NaOH (w/v) at 100°C for 2h followed by the enzymatic pretreatment (Cellulase, 20Ug^{-1}), a hydrogen yield of 300 L H_2 kg^{-1} VS was observed versus 31.4 L H_2 kg^{-1} VS for the enzymatic pretreatment alone (Chairattanamanokorn et al., 2009). Combined acid-enzymatic pretreatments were shown to enhance significantly hydrogen potentials from wheat straw (Nasirian et al., 2011). Indeed, hydrogen potentials of 5.7 L H_2 kg^{-1} VS, 37 L H_2 kg^{-1} VS and 125 L H_2 kg^{-1} VS were respectively observed for untreated, acid pretreated and combined acid-enzymatic pretreated wheat straw (Nasirian et al., 2011). In these cases, chemical pretreatment prior to enzymatic hydrolysis seems to enhance enzymatic accessibility to holocelluloses and further increase hydrogen production of lignocellulosic substrates.

As for **methane potentials**, Ghost and Bhattacharrya (1999) studied the effect of white-rot fungi and brown-rot fungi on rice straw. Increases in methane of 32% and 46% were observed respectively for rice straw pretreated with brown- and white-rot fungi compared to untreated straw (Ghosh and Bhattacharyya, 1999). The use of enzymes to increase methane yield has also been studied (Guiot et al., 2009; Lehtomaki et al., 2004). Lehtomaki et al (2004) applied enzymatic pretreatment to grass at 35°C for 24 h using two xylanases (GC 320 and Multifect) and two cellulases (IndiAge MAX L and Primafast 200). A slight increase, from 230 L CH_4 kg^{-1} VS to 271 L CH_4 kg^{-1} VS, was observed (Lehtomaki et al., 2004). Guiot et al. (2009) noticed that lignolytic enzymes can be benefit to increase methane potentials. Indeed, an energy gain of 29 and 42% were evaluated respectively using lignin peroxidase and manganese peroxidase (Guiot et al., 2009). Recently, Frigon et al. (2012) have shown that using pectinase can improve significantly anaerobic digestion from switchgrass as methane potentials increases of 40 % and 72 % were respectively noticed using polygalacturonase and pectate-lyase enzymes. For methane potentials, combined acid with

enzymatic pretreatments on sugarcane bagasse give promising results as an increase of 208 % of the energy gain was noticed (Badshah et al., 2012).

Ensiling is a biological conservation process that can be used as pretreatment. According to Lehtomaki (2006), ensiling have a positive impact most of the time, and would result in up to 31% methane production from the crops. Bauer et al. (2009) have investigated methane potentials from several energy crop silages and found that methane potentials ranged from 345 L CH_4 kg^{-1} VS (sunflower sillage) to 375 L CH_4 kg^{-1} VS (barley sillage) and the degree of degradation average was of 78.2% (Bauer et al., 2009). Similar results were observed in silages such as maize and grass, with methane production of 370 L CH_4 kg^{-1} VS and 431 L CH_4 kg^{-1} VS respectively (Bruni et al., 2010; Pakarinen et al., 2009). A 25 % increase in methane potential was observed for maize after four months ensiling compared to fresh maize (Neureiter et al., 2005). Similar trend was noticed on hemp ensiled for 4 months with a methane increase over 50 % compared to fresh hemp (Pakarinen, 2012a). However, ensiled faba bean led to less methane than the fresh material (Pakarinen, 2012a). Recently, Herrmann et al. (2011) showed that ensiling prolonged storage and biological commercial silage additives have positive effects on methane yields of up to 11 %. Indeed, biological silage additives normally inhibit or restrict undesirable silage fermentation or aerobic deterioration (Herrmann et al., 2011).

Table I. 9 *Biohydrogen or methane potentials enhancement from biologically (micro-organisms and ensiling) pretreated lignocellulosic residues.*

Categories	Lignocellulosic material	Pretreatment conditions	$BioH_2$ production (L H_2 kg^{-1} VS_{added})	CH_4 production (L CH_4 kg^{-1} VS_{added})	Energy from pretreated biomass [a] (MJ kg^{-1} VS_{added})	Energy from raw biomass [a] (MJ kg^{-1} VS_{added})	Energy gain (%)	References
Micro-organisms	Maize leaves	Aerobic bacterium Bacillus amyloliquefaciens	73.1		0.78	0.18	333	(Ivanova et al., 2009)
	Rice straw	White rot-fungus *Phanerochaete chrysosporium*		328	13.05	8.91	46	(Ghosh and (Bhattacharyya, 1999)
	Rice straw	Brown rot-fungus *Polyporus ostreiformis*		295	11.74	8.91	32	(Ghosh and (Bhattacharyya, 1999)
	Grass hay	White rot-fungus *Pleurotus ostreatus*		240	9.55	9.15	4	(Lehtomaki et al., 2004)
	Corn straw	Microbial agents (0.01 % (w/w)); mixture of yeast, cellulolytic bacteria and lactic acid bacteria, 20 days		222	8.83	5.05	75	(Zhong et al., 2011)
	Wheat straw	Fungus *Pleurotus florida*, 90days		343	13.6	11.65	17	(Muller and Trosch, 1986)
	Japanese cedar chips	Fungus *Cyathus Stercoreus AW 03-72*		43[b]	1.71	0	-	(Take et al., 2006)
	Japanese cedar chips	Fungus *Trametes hirsuta AW 03-72*		30[b]	1.19	0	-	(Take et al., 2006)
	Corn straw	Fungus *Pleurotus florida*, 60days		407[b]	16.19	6.12	164.5	(Zhong et al., 2010)
Ensiling	Maize	Ensiling (4 months)		480	19.09	15.23	25	(Neureiter et al., 2005)
	Hemp	Ensiling (4 months)		380	15.12	9.55	58	(Pakarinen, 2012a)
	Maize	Ensiling (8 months)		445	17.70	15.51	14.1	(Pakarinen, 2012a)
	Faba bean	Ensiling (8 months)		390	10.94	15.51	- 41	(Pakarinen, 2012a)

[a] Energy yield 1 Nm^3 CH_4 = 39790 kJ; 1 Nm^3 H_2 = 10780 kJ
[b] mL CH_4 kg^{-1} TS added or MJ kg^{-1} TS added.

Table I. 10 *Biohydrogen or methane potentials enhancement from enzymatically pretreated lignocellulosic residues.*

Categories	Lignocellulosic material	Pretreatment conditions	$BioH_2$ production (L H_2 kg^{-1} VS_{added})	CH_4 production (L CH_4 kg^{-1} VS_{added})	Energy from pretreated biomass [a] (MJ kg^{-1} VS_{added})	Energy from raw biomass [a] (MJ kg^{-1} VS_{added})	Energy gain (%)	References
Enzymes	Wheat straw	Acid (120°C, 90 min, 2% H_2SO_4 (w/v) + Enzyme Methaplus® (mixture of cellulase, xylanases and β-glucanases)	125		1.34	0.06	2233	(Nasirian et al., 2011)
	Bagasse	100°C, 2h + cellulase (20 FPU g^{-1})	31.3		0.34	-	-	(Chairattanamanokorn et al., 2009)
	Bagasse	100°C, 2h, 4% NaOH (w/v) + cellulase (20 FPU g^{-1})	300		3.23	-	-	(Chairattanamanokorn et al., 2009)
	Poplar leaves	2 % (v/v) viscozyme L	45[b]		0.49[b]	0.16[b]	206	(Cui et al., 2010)
	Wheat straw	Enzyme mixture of cellulase and hemicellulases (5 mg protein. g^{-1} raw wheat straw)	19.6		0.21	0.11	85	(Quemeneur et al., 2012a)
	Fresh summer switchgrass	Mulched (0.2 to 1 cm) with knife mill (GM200) + Lignin peroxidase (20 U g^{-1}TS)		202	8.04	6.20	29	(Guiot et al., 2009)
	Fresh summer switchgrass	Mulched (0.2 to 1 cm) using knife mill (GM200) + Manganese peroxidase (40 U g^{-1}TS)		223	8.87	6.20	42	(Guiot et al., 2009)
	Grass hay	Genencor industrial enymes: Xylanases (GC320, Multifect) + 2 cellulases (Indiage Max L, Primafast) at 0.1 % (w/wTS)		280	11.14	9.15	22	(Lehtomaki et al., 2004)
	Sugarcane bagasse	2 % H_2SO_4 (w/v) / 121°C / 15 min + Enzyme Accelerase® (mixture of cellulase, hemicellulases and β-glucosidase)		200	7.95	2.58	208	(Badshah et al., 2012)
	Fresh summer switchgrass	Polygalacturonase (50 U g^{-1} VSS)		239.5	9.52	5.53	72	(Frigon et al., 2012)
		Polygalacturonase (10 U g^{-1} VSS)		64.9	2.58	5.53	-53	(Frigon et al., 2012)
		Pectate-lyase (6313 U g^{-1} VSS)		287.5	11.42	8.15	40	(Frigon et al., 2012)
		Pectate-lyase (1263 U g^{-1} VSS)		205	7.56	8.15	-7	(Frigon et al., 2012)
	Potatoes	0.1 % (w/w) α amylase + 0.2% (w/w) glucoamylase	271	145	8.69	7.33	18.5	(Xie et al., 2007)

[a] Energy yield 1 Nm^3 CH_4 = 39790 kJ; 1Nm^3 H_2 = 10780 kJ

[b] mL H_2 kg^{-1} TS added or MJ kg^{-1} TS added.

4.4 Impact of pretreatment on two-stage H_2/CH_4 process

Few papers investigated pretreatment prior to two-stage H_2/CH_4 process (Lu et al., 2009, Pakarinen et al., 2009, Pakarinen et al., 2011; Xie et al., 2007). Among them, some studies concerned very low lignin-content substrates such as potatoes (Xie et al., 2007). Xie et al., (2007) have investigated an enzymatic pretreatment (0.1 % (w/w) α amylase + 0.2% (w/w) glucoamylase) on potatoes before two-stages H_2/CH_4. Hydrogen and methane production of 271 L H_2 kg^{-1} VS and 145 L CH_4 kg^{-1} VS were repectively observed corresponding to an energy gain increase of 18.5% compared to one-stage CH_4 process

Lu et al. (2009) studied steam explosion of cornstalks prior to two-stage process but they did not indicate bioH_2 and CH_4 production from the untreated substrate. Low hydrogen and methane production of respectively 7.8 L H_2 $kg^{-1}_{cornstalks}$ and 13.1 L CH_4 $kg^{-1}_{cornstalks}$ were observed. If the hydrogen production is in accordance with the literature on the same or equivalent substrates, the methane potential remained very low (Zhang et al., 2006; Quemeneur et al., 2012a). One explanation is that batch anaerobic stage was performed only during 9 days and thus a large part of the substrate was not degraded. Lu et al. (2009) have also investigated an original three-stage process with an hydrolysis step (pH=5.5) prior to hydrogen and methane steps with pH of 6.5 and 7.5 respectively. This hydrolysis step before two-stage process has permitted to enhance significantly both hydrogen and methane production with respectively 19.5 L H_2 $kg^{-1}_{cornstalks}$ and 31.6 L CH_4 $kg^{-1}_{cornstalks}$.

Few studies compared the efficiency of pretreatment on two-stage bioH_2 and CH_4 production in comparison with one-stage CH_4 production. Pakarinen et al. (2009) investigated the impact of alkaline pretreatment (4% NaOH, 20°C, 24 h) on a two-stage process. A 16% increase in bioH_2 production but no significant increase in CH_4 production was observed (Pakarinen et al., 2009). However, this study was carried out with grass sillage and, as mentioned above, sillage can be considered as a biological pretreatment and may have lowered the impact of the alkaline pretreatment. In another study, Pakarinen et al., (2011) have investigated two pretreatments (water extraction and HCl) to enhance two-stage H_2/CH_4 process from maize. Increases in CH_4 yields of 7 %, 9 % and 27 % were respectively noticed for untreated, water extracted and HCl-treated maize with two-stages process (H_2/CH_4) compared to one-stage process (Pakarinen et al., 2011).

Recently, Cheng and Liu (2011b) applied alkaline pretreatment on cornstalks to enhance two-stage process H_2/CH_4 conversion efficiency. After alkaline pretreatment, the dried solid residues were used for batch hydrogen fermentation and subsequent batch methane production. The liquid hydrolyzate after alkaline pretreatment was directly used for batch methane production. An increase of hydrogen potential from 37.6 L H_2 kg^{-1} VS to 45.7 L H_2 kg^{-1} VS and methane potential from 112 L CH_4 kg^{-1} VS to 164 L CH_4 kg^{-1} VS was noticed (Cheng and Liu, 2011b).

Two-stage H_2/CH_4 has also been investigated in biorefinery concept (Kaparaju et al., 2009; Luo et al., 2011). Kaparaju et al. (2009) tested an original three-stage process on wheat straw, producing bioethanol, biohydrogen and biomethane. Initially, wheat straw was hydrothermally pretreated, giving a cellulose-rich solid fraction and a hemicelluloses-rich liquid fraction (hydrolysate). After enzymatic hydrolysis of the solid fraction, an ethanol yield of 0.41g ethanol g^{-1} sugars was observed and a biohydrogen yield of 178 mL H_2 g^{-1} sugars from the liquid fraction. The effluents from both the bioethanol and biohydrogen processes were further used to produce methane, with yields of 324 and 381 mL g^{-1} VS added, respectively (Kaparaju et al., 2009). Luo et al. (2011) have investigated an original four-stage from rapeseed plant in a biorefinery concept, producing, biodiesel, bioethanol, biohydrogen and biomethane. First, rapeseed plant was seprated into oil seed and rapeseed straw respectively for the production of biodiesel and bioethanol. Biodiesel was produced on oil seed by oil extraction process follow by transesterification and lead to the production of two byproducts as cake and glycerol. Bioethanol was produced from rapeseed plant by a pretreatment (combination of alkaline with steam pretreatment) follow by enzymatic hydrolysis and fermentation. Such process lead also to the generation of byproducts as stillage and hydrolyzate. Both byproducts of biodiesel and bioethanol were further used to produce biohydrogen and methane in a two-stage H_2/CH_4. This biorefinery concept lead to an energy recovery of 60 % of the total energy obtained by combustion of rapeseed plant (240 GJ/ (ha a)). Even if this recovery is less important than direct combustion, conversion of biomass to energy carriers (biodiesel, bioethanol, biohydrogen and methane) is energetically more positive with respect to power production, since internal combustion engines have higher power generation efficiency than a boiler and steam cycle, which is also very costly in small case (Luo et al., 2011). However, most of these studies have been performed in batch assays and data on continuous reactors are thus missing to assess long term productivity and feasibility at industrial scale. Moreover, continuous

reactors are compulsory to establish accurate economic and energetic balances of two-stage H_2/CH_4 compared to one-stage CH_4 with and without pretreatment.

4.5 General remarks

Table I.11 summarizes the main effects of different pretreatment categories on compositional and structural features of lignocellulosic biomass. All pretreatments were found efficient in increasing the accessible surface by gradual removal of lignin, hemicelluloses, pectin or cellulose constituents. Fungal and enzymatic pretreatments were found effective in lignin, cellulose and hemicelluloses removal according to the fungal strains or enzymes used. However, the rates of biological hydrolysis are usually very low, so pretreatment using fungi requires long residence times of several days. Ionic liquid, mechanical and AFEX pretreatments were found efficient in reducing significantly the crystallinity of cellulose. Among thermo-chemical pretreatements, oxidative and basic pretreatment are efficient in lignin removal whereas acid pretreatments are more efficient in solubilisation of hemicelluloses.

However, when pretreatment conditions are too severe, especially in the case of physical pretreatment at high temperature (LHW, steam explosion) and thermo-chemical pretreatment that requires high temperatures (acid or organosolv), some byproducts such as furfural, hydroxymethylfurfural and soluble lignin compounds can be generated (Palmqvist and Hahn-Hägerdal, 2000). The accumulations of such compounds become a problem during the fermentation stage of bioethanol production because they inhibit, or even stop, the fermentation stage (Laser et al., 2002). In contrast to the bioethanol process, anaerobic digestion can convert these compounds. However, the methanogenic microorganisms require a period of adaptation decreasing the methane production rate but not the final methane yield (Benjamin et al., 1984; Fox et al., 2003, Barakat et al., 2012). On the contrary, biohydrogen production was found highly influenced by the presence of these byproducts (furfural, 5-HMF and phenolic compounds). Quemeneur et al., (2012b) showed a negative impact on hydrogen production in mixed cultures from xylose when furans and phenolic compounds were added individually at a concentration of 1g L^{-1}.

Table I.11 summarizes also the state of the art of the use of different pretreatment categories to enhance both hydrogen and methane production from lignocellulosic residues. A large range of

pretreatments have been investigated to enhance methane production and less for hydrogen production. Pretreatments were found to enhance with more or less success hydrogen and methane potentials .Among thermo-chemical pretreatments, hydrogen potentials enhancement varied from 2.8 % (alkaline pretreatment on grass sillage) to 5300 % (acid pretreatment on corn stalks) whereas methane production enhancement ranged from 17 % (alkaline pretreatment on grass hay) to 792 % (alkaline pretreatment on barley waste). Moreover, in some cases pretreatment resulted in no increase or decrease of hydrogen or methane yields.

Acid pretreatment was shown to be advantageous for hydrogen production whereas thermo-chemical (steam explosion, wet oxidation) and alkaline pretreatments are better for methane production. As well by increasing biodegradability and, thus, biohydrogen and methane production, pretreatment is sometimes effective in increasing the hydrolysis rate of lignocellulosic biomass. In this case, solid retention time can be lowered in the digester, making for a higher organic load and a consequent increase in methane production in a given digestor. For example, Fernandes et al. (2009) measured first-order hydrolysis rate of anaerobic digestion with hay pretreated with 4 % ammonium (w/v). No significant increase in methane potential was observed but the hydrolysis rates increased from 0.088 to 0.409 d^{-1} (Fernandes et al., 2009).

Table I. 11 *Main effects of pretreatments on structural and compositional features of lignocelluloses substrates and state of pretreatment research for hydrogen and methane productions(adapted from Mosier et al., 2005).*

	PHYSICAL				**THERMO-CHEMICAL**		
	Milling	**Microwave**	**Liquid hot water**	**Steam explosion**	**AFEX**	**CO_2 explosion**	**Wet oxidation**
Compositional and structural changes	• Increase accessible surface area • Reduction of crystallinity	• Increase accessible surface area • Partial solubilisation of holocelluloses • Formation of byproducts from lignin or sugars degradation	• Increase accessible surface area • Solubilisation of hemicelluloses • Formation of byproducts from lignin or sugars degradatin	• Increase accessible surface area • Solubilisation of hemicelluloses	• Increase accessible surface area • Reduction of crystallinity • Solubilisation of lignin	• Increase accessible surface area • Solubilisation of hemicelluloses • Alteration of lignin -• Reduction of crystallinity	• Increase accessible surface area • Solubilisation of holocelluloses.
State of research for hydrogen/methane	+ / ++	+ / +	Not found / ++	+ / ++	Not found / Not found	Not found / Not found	Not found / +

	Thermo-chemical					Biological	
	Oxidative	**Alkaline**	**Acid**	**Organosolv**	**Ionic liquid**	**Micro-organisms**	**Enzymes**
Compositional and structural changes	• Increase accessible surface area • Partial solubilisation of hemicelluloses • Formation of byproducts from lignin or sugars degradation at high temperature	• Increase accessible surface area • Solubilisation of lignin • Patial solubilisation of hemicelluloses • Formation of byproducts from lignin or sugars degradation at high temperature	• Increase accessible surface area • Solubilisation of hemicelluloses • Partial solubilisation of cellulose • Formation of byproducts from lignin or sugars degradation • Alteration of lignin	• Increase accessible surface area • Solubilisation of hemicelluloses • Solubilisation of lignin • Formation of byproducts from lignin or sugars degradation	• Increase accessible surface area • Reduction of crystallinity • Solubilisation of lignin and cellulose	• Increase accessible surface area • Solubilisation of lignin • Partial solubilisation of holocelluloses.	• Increase accessible surface area • Solubilisation of hemicelluloses (xylanase) • Solubilisation of cellulose (cellulase) • Solubilisation of lignin (lignolytic enzyme) • Solubilisation of pectin (pectinases)
State of research for hydrogen/methane	+ / +	++ / ++	++ / ++	Not found / Not found	Not found / +	+ / +	+ / +

+ : little investigated; ++: well investigated

5. Conclusions

The present literature overview reports recent findings on pretreatments to enhance biohydrogen and methane from lignocellulosic residues. The main advantage of these two processes compared to bioethanol or biodiesel production is that they use consortia of microorganisms which accept a large range of substrates (cellulose, hemicelluloses, lipids, proteins...). Moreover, biohydrogen and biomethane production can be combined in a two-stage process in which biohydrogen is produced in a first reactor by H_2 dark fermentation and methane is produced in a second reactor using the soluble metabolites of dark H_2 fermentation. The gas mixture (hythane: H_2/CH_4) presents several advantages as it can significantly reduce CO_2 and other emissions and improve fuel combustion efficiency.

However, due to the poor accessibility of biodegradable compounds embedded within the lignocellulosic matrix, pretreaments are required to enhance both hydrogen and/or methane production. If in the case of bioethanol production, pretreatment strategies have for main objective to solubilise lignin and hemicelluloses to make cellulose more accessible, pretreatments strategies for biohydrogen and methane have not been well defined yet. A large range of pretreatments have been investigated with more or less success to enhance biohydrogen and methane production from lignocellulosic substrates and in some cases no increase or decrease of biohydrogen and methane production were observed after pretreatment. Pretreatment are often discussed on their enhancement of hydrogen and methane potentials without focus on biochemical and structural changes of lignocellulosic matrix induced by pretreatment. Thus, further research on chemical and structural features limiting the lignocellulosic conversion are necessary to optimize pretreatment strategies. Moreover, once pretreatments strategies defined and optimized, they should be validated in continuous reactors to be close to the industrial scale and establish energetic balances. In addition, the choice of a pretreatment method should not be based only on its energy gain but should also consider parameters like economic cost and environmental impact. Indeed, economic models show that pretreatments is an essential operation requiring a dedicated unit accounting for 16-19 % of total cost equipment of the lignocellulosic biorefinery (Aden et al., 2009).

Chapitre II. Materials and methods

1. Lignocellulosic materials and preparation

Twenty lignocellulosic substrates were used to perform all experimental tests. All substrates were considered as lignocellulosic materials, except for Jerusalem artichoke tuber, which is a substrate rich in inulin (polymers of fructose) that can be used for first generation biofuels. Some lignocellulosic substrates were chosen because they represent agricultural residues which have few suitable end-uses and are generally burnt in the fields causing environmental pollution. Moreover, these substrates present the advantage to be renewable, in abundance and cost free. Energy crops like biomass sorghum, forage sorghum and sweet sorghum were also investigated as they can be cultivated in soils unsuitable for food production and represent interesting feedstock for the production of biofuels, due to their high content in sucrose. Above all, all these substrates were chosen because they have very different chemical composition and thus cover a large range of compositional and structural features. All these lignocellulosic substrates were cultivated in South of France. These substrates were classified into two categories: **agricultural residues** such as rice straw, giant reed (stalks and leaves), three varieties of sunflower stalks, sunflower bark, sunflower oil cakes, maize (stalks, leaves and cobs), Jerusalem artichoke (stalks, leaves and tubers) and seed sorghum stalks (sorghum 1) and **energy crops** such as biomass sorghum (sorghum 2), forage sorghum (sorghum 4) and sweet sorghums, (sorghums 3, 5, and 6).

Except for Jerusalem artichoke tubers and sorghum 2, 3, 4, 5 and 6, all substrates were first milled into 2 mm particles size, by using a cutting milling (SM 100, Restch) and then dried at 37°C for 48 h. All sorghum varieties were stored at -20°C and dried with a freeze drying (HetoPowerDry PL 3000; ThermoElectron Corporation). They were further milled into 2 mm particles size, by using a cutting milling (SM 100, Restch).

Jerusalem artichoke tubers were first washed with tap water, to remove the soil fractions remaining on the substrate, and then they were stored at -20°C and dried with a freeze drying (HetoPowerDry PL 3000, ThermoElectron Corporation). They were further milled into 2 mm particles size, by using a cutting milling SM 100.

All substrates were conserved into airtight vessels at ambient temperature. The substrates were analyzed for Total Solids (TS) and Volatile Solids (VS) contents according to the APHA standard method described in part 4.1.

Among the substrates described before, sunflower stalks were selected to apply pretreatments (thermo-chemical, enzymatic or combination of them) and anaerobic fermentation for biohydrogen and methane production. However, in reason of limited amounts of substrates and limited storage time, four different samples were used: NK-Kondi, Serin 1, Serin 2 and Naturasol. Sunflower stalks Serin 1 and Serin 2 belong to the same variety but they were not grown at the same place and their storage conditions were different. Indeed, Serin 1 and Serin 2 stalks were collected two weeks and two months after harvest, respectively.

Table II.1 summarizes the variety of sunflower stalks and their preparation (milling), according to the type of pretreatment and the fermentation process used.

Table II. 1 *Sunflower stalks varieties used and their preparation according the experiments.*

Pretreatment	Fermentation process	Chapters	Variety	Preparation
Thermo-chemical pretreatments	Methane, batch	IV.2, VI.2	"NK-Kondi"	milling (2mm)
Alkaline pretreatments: impact of parameters	Methane, batch	VI.3	"NK-Kondi; Serin 1"	milling (2mm)
Alkaline pretreatments: varieties of sunflower stalks	Methane, batch	VI.4	"NK-Kondi; Serin 1; Serin 2; Naturasol"	milling (2mm)
Combined alkaline-enzymatic pretreatments	Hydrogen, batch	IV.3, V.3	"Serin 1"	milling (2mm)
Dilute acid pretreatments	Hydrogen, batch	V.2	"Serin 1"	milling (2mm)
Alkaline pretreatments	Methane (continuous)	VII	"Serin 1"	Milling (0.5mm)

2. Pretreatment methods

2.1 Thermo-chemical pretreatments

Various kinds of thermo-chemical pretreatments (NaOH, H_2O_2, Ca $(OH)_2$, HCl and $FeCl_3$) were tested on sunflower stalks with a solids load of 35 g TS L^{-1}. First a general protocol for the different thermo-chemical pretreatments used is presented. In a second part, the operational conditions for each pretreatment according the different chapter are described.

NaOH, H_2O_2, Ca $(OH)_2$ pretreatments were carried out in 600 mL flasks. HCl and $FeCl_3$ pretreatments were performed in Zipperclave autoclave series 02-0378-1 (Autoclave France). This autoclave, in stainless steel with a capacity of 1 L, can reach a temperature of 250 °C and a pressure of 79 bars. The reactor content was agitated by a rod with two propellers at a rate of 150 rpm and was heated by a ceramic furnace. All pretreatment conditions depending on the experiments are presented in Table II.2; Table II.3 and Table II.4.

In chapter IV and VI, NaOH, H_2O_2, Ca $(OH)_2$ pretreatments were carried out in 600 mL flasks with chemical agent concentration of 4% (g/ 100g TS) for 24 hours at 55°C. Thermal pretreatment alone at 55°C was also carried out. These assays were performed using an "Edmund Butler" heating shaker series SM-30-control with an agitation of 150 rpm. Two bottles with a working volume of 57 mL were performed for further Biochemical Methane Potentials (BMP) tests. One control with a working volume of 200 mL was also realized. At the end of pretreatment, the hydrolysate of the control bottle was separated from the solid fraction by filtration through a sieve of 0.25 mm of pore size. The hydrolysates were kept at 4°C for further chemical analyses and solids fraction were washed several times with water to remove water soluble compounds and dried at 60°C for 24 h. Then, the solid fraction was kept in a hermetic vessel for further chemical and FT-IR analysis.

HCl and $FeCl_3$ pretreatments were carried out at 170°C with concentration of 4% (g/ 100g TS) and 10% (g/ 100g TS), respectively. Thermal pretreatment alone at 170°C was also carried out. The working volume used during the experiment was of 400 mL. Then, two Biochemical Methane Potential assays were carried out by taking 57 mL of each pretreated sample. The remaining part of pretreated samples was further filtrated through a sieve of 0.25 mm of pore size to separate the hydrolysate from the solid fraction. The hydrolysate was kept at 4°C for further chemical analyses and solids fractions were washed several times with water to remove water

soluble compounds and dried at 60°C for 24 h. The solid fraction was kept in a hermetic vessel for further chemical and FT-IR analysis.

Table II. 2 *Operating conditions of thermo-chemical pretreatments in chapter IV and VI.*

Chemical reagent	Temperature (°C)	Concentration (g/ 100g TS)	Time	pH intial
No chemical	55	-	24	7.5
No chemical	170	-	1	7.5
NaOH	55	4	24	11.9
$Ca(OH)_2$	55	4	24	12.2
H_20_2 (pH=11.5, adjusted with NaOH)	55	4	24	11.6
HCl	170	4	1	2.3
$FeCl_3$	170	10	1	1.8

In chapter VI, the impact of pretreatment parameters, i.e. time, temperature, sodium hydroxide concentration, on methane potentials was investigated (Table II.3). Three temperatures (30°C, 50 °C and 80 °C) were investigated at fixed concentration (4 g/100gTS) and fixed time (24 h). Then five different times of pretreatment (3, 6, 12, 24 and 36h) were tested at fixed temperature (55°C) and fixed concentration (4 g/100gTS). To finish, five sodium hydroxide concentrations (0.5, 2, 4, 6 and 10 g/100gTS) were investigated at fixed temperature (55°C) and fixed time (24 h). For each condition, two bottles with a working volume of 57 mL were performed for further Biochemical Methane Potentials (BMP) tests. These assays were performed on an "Edmund Butler" heating shaker series SM-30-control with an agitation of 150 rpm.

Table II. 3 *Operating conditions of alkaline pretreatments of chapter VI.*

Temperature (°C)	Concentration (g/ 100g TS)	Time
30; 55; 80	4	24
55	4	3; 6; 12; 24; 36
55	0.5; 2; 4: 6: 10	24

Then, the condition (55°C, 24 h, 4% NaOH (g/gTS)) was investigated on four varieties of sunflower stalks NK-Kondi, Serin 1, Serin 2 and Naturasol. For each variety of sunflower stalks, two bottles with a working volume of 57 mL were performed for further Biochemical Methane Potentials (BMP) tests.

In the chapter V, the effect of dilute-acid pretreatment parameters, i.e. temperature and concentration, on biohydrogen production from sunflower stalks was investigated using a central composite design. To build the central composite design, values for the two parameters (temperature and acid concentration) were centred and scaled, which led to new variables called "coded values". Correlations between coded and real values used in the composite design are presented in Table II.4. The working volume used during the experiment was of 400 mL for each condition. Then, two Biochemical Methane Potential assays were carried out by sampling 57 mL of each pretreatment.

Table II. 4 *Coded and real values used to build the experimental composite design in chapter V.*

Coded values	Real values	
	X1: Temperature (°C)	X2: HCl concentration (g / 100g TS)
-1.21	142	0
-1	150	0.6
0	170	2
1	190	3.4
1.21	198	4

In the chapter VII, alkaline pretreatment (55°C, 24 h, 4% NaOH (g/gTS)) was daily carried out in 600 mL bottles with a working volume of 100 mL. Bottles were continuously agitated for complete mixing by using a magnetic stirrer, and maintained at 35°C by a bath water.

2.2 Combined alkaline and enzymatic pretreatments

Alkaline pretreatments were performed as previously described in part 2.1. In this case, two 600 mL bottles with a working volume of 100 mL were dedicated to further biohydrogen chemical potentials tests. A third bottle, with a working volume of 400 mL, was further filtrated through a sieve of 0.25 mm of pore size to separate the liquid and the solid fractions. The liquid fraction samples (liquors) were kept at 4°C for further biohydrogen chemical potentials. Solid fractions were washed several times to remove water soluble compounds and dried at 60°C for 24 h. Then enzymatic pretreatments were performed on raw sunflower stalks and on the solid residue of alkaline-pretreated sunflower stalks. For this, untreated and solid residues from alkaline pretreated (55°C, 24 h, 4% NaOH) sunflower stalks were first autoclaved during 20 min at 121°C (SYSTEC VB 150). Indeed, in the case of enzymatic pretreatment, aseptic conditions or free oxygen

conditions are needed to avoid the consumption of free sugars by indigenous microorganisms present on the lignocellulosic substrates (Quemeneur et al., 2012a). Enzymatic hydrolysis of the untreated and solid alkaline pretreated sunflower stalks was performed at a solid loading of 50 g TS L^{-1} in flasks of 600 mL incubated at 35°C in an "Edmund Butler" heating shaker series SM-30-control with an agitation speed of 150 rpm. The working volume was 70 mL and the pH was fixed at 5, by using 50 mM of 2-(N-morpholino) ethanesulfonic acid (MES) buffer. Four replicates were realized: two bottles for further Biochemical Hydrogen Potential tests and two control bottles for further chemical analysis. Cellulase from *T.reesei* (Sigma-Aldrich Corporation) was added at a concentration of 50 FPU g^{-1} TS of the untreated and washed pretreated solid. β-glucosidase (Sigma-Aldrich Corporation) from *Aspergillus niger* (Sigma-Aldrich Corporation) and xylanase from *Thermomyces lanuginosus* (Sigma-Aldrich Corporation) were added at, respectively, 25 U g^{-1} TS and 50 U g^{-1} TS. After 48 h of enzymatic hydrolysis, 2 mL of the control bottles were taken, placed in hermetic Pyrex vessels and put at 105°C during 5 min to stop the enzymatic activity. The samples were further centrifuged at 5000 g during 10 min (Eppendorf, Mini spin) followed by filtration at 0.2 µm (Nylon membrane, Acrodlsc®). Then, 800 µL of supernatant were taken to the analysis of monomers sugars (xylose and glucose) released during enzymatic hydrolysis by high-pressure liquid chromatography (HPLC).

2.3 Dilute acid pretreatments: impact of hydrolysate concentrations

Dilute acid pretreatment at 170°C, 4% HCl during 1 hour was carried out and at the end of pretreatment; the hydrolysate was separated from the solid fraction by filtration through a sieve of 0.25 mm of pore size. Then, the effect of different added volumes (3.75%, 7.5%, 15% and 35% (v/v)) of dilute-acid hydrolysate (170°C, 4% HCl) on biohydrogen production from glucose at 5 g L^{-1} was investigated (Figure II.1).The remaining hydrolysate was stored at 4°C for chemical analyses of metabolites and byproducts (furfural, 5-HMF and phenolic compounds) of lignocellulosic degradation.

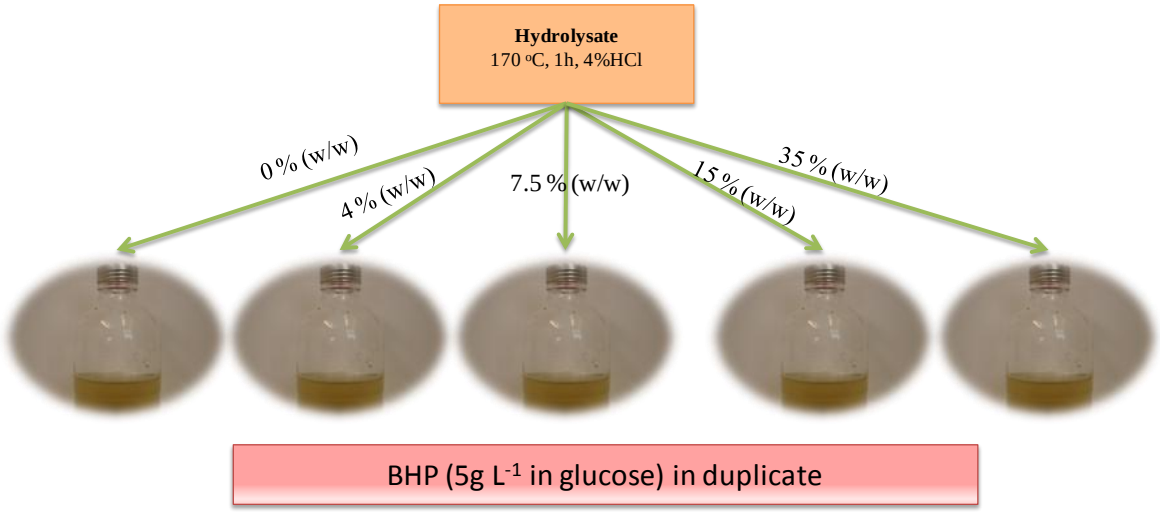

Figure II. 1 *Biochemical Hydrogen Potential (BHP) tests at 5 g glucose L^{-1} with different added volumes (3.75%, 7.5%, 15% and 35% (v/v)) of dilute-acid hydrolysate (170°C, 4% HCl).*

3. Biological processes used for biohydrogen and methane production

3.1 Biological Hydrogen Potential (BHP) test

Biohydrogen potential batch experiments were carried out in batch mode at 37 °C without agitation (Figure II.2). BHP tests were realized in flask of 600 mL, with a working volume of 200 mL or in a reactor of 5.5 L with a working volume of 4.5 L for the last experiment where two stages H_2/CH_4 was investigated. A quantity of raw substrate and of pretreated samples was initially introduced in each flask according the experiment (Table II.4). Then, 200 mL of MES (2-[N-morpholino] ethane sulfonic acid) buffer varying from 50 to 100 mmol L^{-1} (Table II.5).

Table II. 5 *Quantity of substrate (g TS L^{-1}) and concentration of MES buffer (mmol L^{-1}) for Biochemical Hydrogen Potnetial (BHP) tests.*

Experiments	Chapters	Quantity of substrate (gTS L^{-1})	Concentration MES buffer (mmol L^{-1})
Models on compositional and structural features	III.3	15-25	50
Dilute acid pretreatments	VI.2.1	17.5	100
Enzymatic pretreatments	VI.3.1	17.5	100
Dilute acid pretreatments, effect of inhibitors	VI.2.3	5 g glucose	100
Two-stage H_2/CH_4 process	VII	35	0

The inoculum was added to obtain a ratio Substrate/Inoculum around 20 g TS substrate g^{-1} VS inoculum. The inoculum was granular sludge from a mesophilic anaerobic digester of a sugar factory. It was previously heat-treated at 90°C for 15 minutes to inhibit the activity of methanogens and to enrich in hydrogen producing bacteria. No additional nutrient medium solution was added as no effect was observed in previous experiment after adding nutrient on hydrogen production from glucose (Guo et al., 2012). The initial pH value was adjusted to 5.5 with NaOH 2 N or 37 % HCl. The headspace of the flasks was flushed with nitrogen gas to reach anaerobic conditions. Each experiment was performed in duplicate. In the case of enzymatic pretreatment, a blank was realized with only the enzymatic mixture to determine the eventual production of hydrogen from the enzymatic mixture.

Biogas volume was monitored by measuring headspace pressure with a pressure gauge (Mano 2000, Leo 2 Keller).Volume of biogas was deducted from the ideal gas law. The gas composition (O_2, CO_2, CH_4, H_2 and N_2) was analysed using a gas chromatograph (Clarus 580, Perkin Elmer) equipped with two columns, a column (RtQBond) and a molecular sieve (Molsieve, 5Å) and a thermal conductivity detector (TCD). One column (RtQBond) was used to separate H_2, O_2, N_2 and CH_4, and the second one (RtMolsieve) was used to separate CO_2 from other gases. The calibration was carried out with a standard gas (Linde ^{TM}) composed of 25 % CO_2, 2 % O_2, 10 % N_2 and 5 % H_2 and 58 % CH_4. All the values of hydrogen potentials are expressed in standard conditions (0°C, 1013hPa). 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

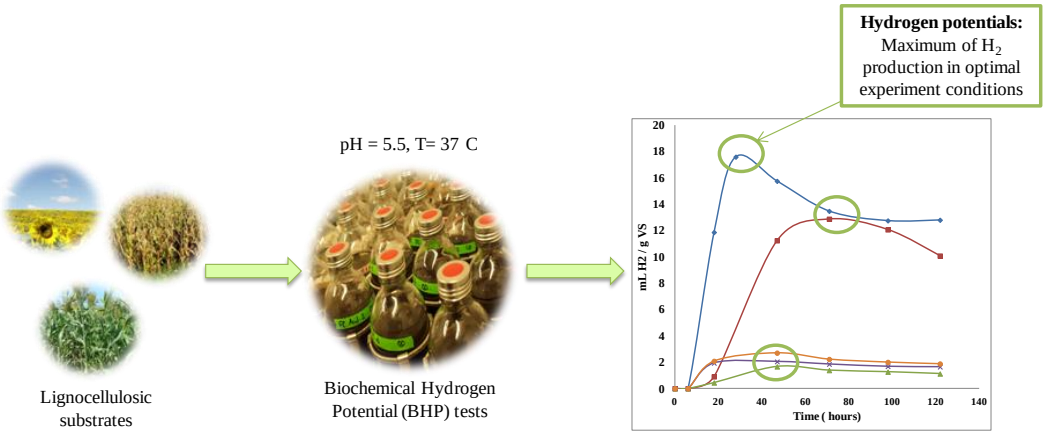

Figure II. 2 *Schematic representation of the biochemical hydrogen potential (BHP) tests.*

3.2 Biological Methane Potential (BMP) test

Lignocellulosic substrates pretreated or untreated were digested anaerobically in batch anaerobic flasks at 37 °C without agitation (Figure II.3). The volume of each flask was 600 mL, with a working volume of 400 mL. Each flask contained: macroelements (NH_4Cl, 26 g L^{-1}; KH_2PO_4, 10 g L^{-1}; $MgCl_2$, 6 g L^{-1}; $CaCl_2$, 3 g L^{-1}), oligoelements ($FeCl_2$, 2 g L^{-1}; $CoCl_2$, 0.5 g L^{-1}; $MnCl_2$, 0.1 g L^{-1}; $NiCl_2$, 0.1 g L^{-1}; $ZnCl_2$, 0.05 g L^{-1}; H_3BO_3, 0.05 g L^{-1}; Na_2SeO_3, 0.05 g L^{-1}; $CuCl_2$, 0.04 g L^{-1}; Na_2MoO_4, 0.01 g L^{-1}), bicarbonate buffer ($NaHCO_3$, 50 g L^{-1}), an anaerobic sludge at 5 g VS L^{-1} and the substrate untreated or pretreated at 5 g TS L^{-1}. Once the flasks were prepared, a degasification step with nitrogen gas was carried out to obtain anaerobic conditions. The initial pH value was adjusted to 7 with NaOH 2 N or 37 % HCl. The bottles were closed with air impermeable red butyl rubber septum-type stoppers. Each experiment was carried out in duplicate. Assays with only inoculum (completed with distilled water) were used as a control to determine the endogenous production. The inoculum used was granular sludge from a mesophilic anaerobic digester of a sugar factory.

Biogas volume was monitored with a water displacement method daily the first fifteen days and after two times per week. Acidified water (pH =2) was used to minimize dissolution of carbon dioxide. All volumes were expressed in temperature and pressure standard conditions. Biogas composition was determined using a gas chromatograph (Varian GC-CP4900) equipped with two columns: the first (Molsieve 5A PLOT) was used at 110°C to separate O_2, N_2, CH_4, the second (HayeSep A) was used at 70°C to separate CO_2 from other gases. The injector temperature was 110°C and the detector 55°C. The detection of gaseous compounds was done using a thermal conductivity detector. The calibration was carried out with a standard gas composed of 25 % CO_2, 2 % O_2, 10 % N_2 and 63 % CH_4. All the values of methane potentials are expressed in standard conditions (0°C, 1013 hPa).

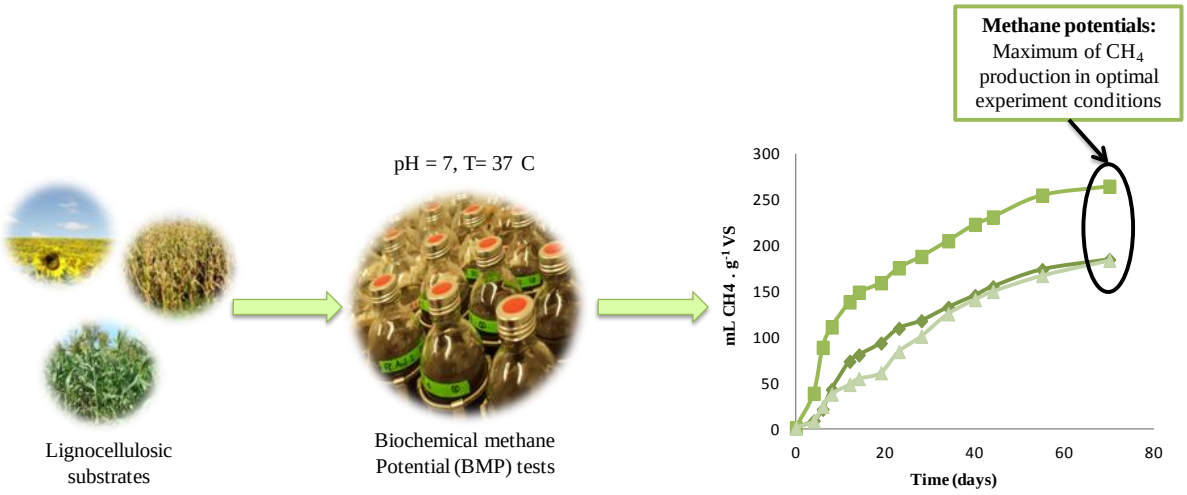

Figure II. 3 *Schematic representation of the biochemical methane potential (BMP) tests.*

3.3 Continuous reactors in one-stage and two-stage processes

In this part, one-stage CH_4 continuous process was investigated in mesophilic conditions with or without alkaline pretreatments, as well as the two-stage H_2/CH_4 process. The three configurations are described in Figure II.4.

The three anaerobic digesters for methane production were performed with identical reactors of 1.5 L of working volume. The reactors were continuously agitated for complete mixing by using a magnetic stirrer, and maintained at 35°C by an external water recirculation system. All the reactors were initially inoculated with a granular sludge from a mesophilic anaerobic digester treating the effluent from a sugar factory, with an initial volatile solid concentration of 50 g VS L^{-1}. Then, they were fed manually and daily five days per weeks with 2.07 g VS L^{-1} d^{-1} corresponding to an organic loading rate (OLR) of 1.49 g VS L^{-1} d^{-1}. Each digesters were fed during three hydraulic retention times (HRT) of 21 days each. The reactors can be considered as "continuous" reactors because their HRT is far longer than the time between two feeding. After two HRTs, each anaerobic reactor was supplemented with 10 mL per week of a NH_4CL solution (30 g L^{-1}).

In the first configuration the digester 1 was fed with raw "Serin" sunflower stalks and digester 2 was fed with pretreated (4% NaOH, 55°C, 24 h) sunflower stalks. For this purpose, the pretreatment was performed every day, according to the protocol defined in part 2. In the configuration 3, (two-stage H_2/CH_4 process), the digester was fed with the outlet of H_2 stage of raw sunflower stalks. Each week, TS, VS, pH and VFAs were

monitored on the three configurations as described in part 4. Biogas produced from the three digesters was collected in 10 L gas bags (Environnmental Samply Supply®). Biogas volume was monitored by using a water displacement method and its composition by gas chromatography (Clarus 580, Perkin Elmer) as described in part 3.1.

In the two-stage H_2/CH_4, H_2 stage for sunflower stalks was performed in batch reactor of 5.5 L with a working volume of 4.5 L. The initial pH value was adjusted to 6 with NaOH 2 N. Gas volume was monitored by water displacement method and biogas composition determined by gas chromatograph (Clarus 580, Perkin Elmer) as described in part 3.1. Once, the cumulative hydrogen production reached its maximum, the outlet of dark fermentation was characterized for metabolites content and further conserved at 4°C for daily feeding of anaerobic digesters 3. All the values of hydrogen and methane potentials are expressed in standard conditions (0°C, 1013hPa).

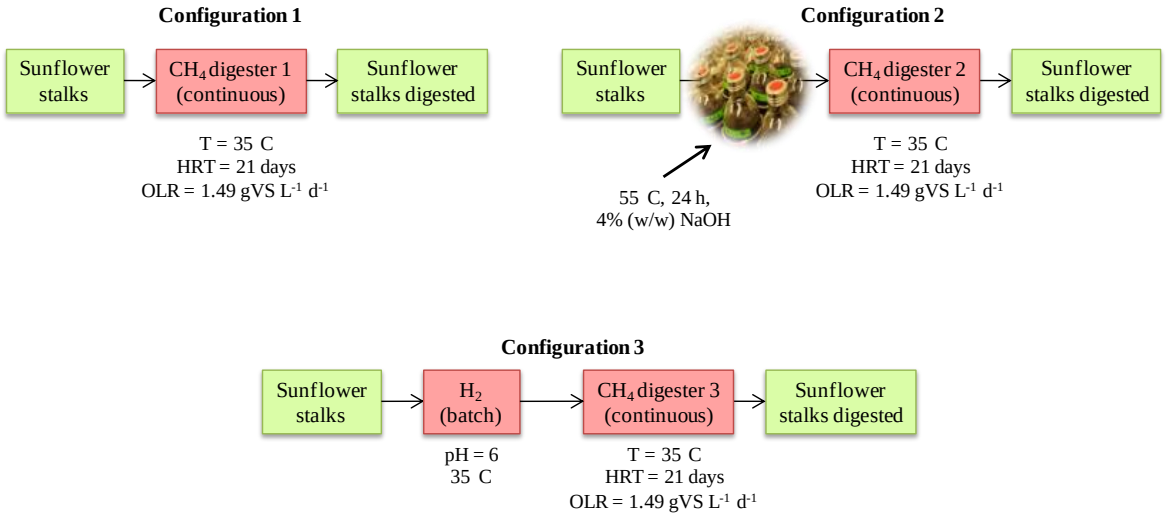

Figure II. 4 *Different configurations of continuous anaerobic digesters.*

4. Analytical methods

4.1 Total solids (TS) and volatile solids (VS)

Porcelain crucibles were placed in an oven at 105°C for at least one hour. They were then placed into desiccators until cooling at room temperature, and then weighted. Around 2 g of each sample was added in the porcelain crucibles, then weighed, and dried in an oven at 105°C for 24 h. 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 24 h drying and the initial sample weight (APHA- 1998). 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- 1998).

4.2 Determination of carbohydrates and Klason Lignin content

Easely **Soluble sugars** (glucose and fructose) from starch, sucrose and inulin were extracted using a mild acid hydrolysis method (Nguyen et al., 2009). Samples (200 mg) were hydrolyzed at 121 °C, 1h, 0.2% H_2SO_4 (w/w) (0.002 M H_2SO_4). After centrifugation of the samples in 2 mL Eppendorf® tubes , followed by filtration at 0.2 µm (Nylon membrane, Acrodlsc ®), 800 µL of supernatant were transferred to a vial prior to the analysis by high-pressure liquid chromatography (HPLC). Soluble carbohydrates (glucose and fructose) were quantified by High-Pressure Liquid Chromatography (HPLC) method coupled to refractometric detection (Waters R410). The analytical chain was composed of an automatic sampler (Water 717plus), a pumping system (DIONEX UltiMate 3000), an oven (DIONEX ultimate 3000RS) and a Biorad HPX-87P column at 85 °C. The eluent corresponded to deionized water under a flow rate of 0.6 mL min^{-1}. The system was calibrated with glucose and fructose standards (0-10g L^{-1}) (Sigma–Aldrich®).

The **structural-carbohydrates** (glucose, xylose, arabinose, uronic acids) content from cellulose, hemicelluloses and pectins was measured using a strong acid hydrolysis method adapted from Effland et al. (1977). Samples (200 mg) were first hydrolyzed with 12 M H_2SO_4 acid for 2 h at room temperature, then diluted to reach a final acid concentration of 1.5 M and kept at 100°C for 3 h. The insoluble residue was separated from the supernatant by filtration on fibreglass paper (GFF, WHATMAN). This insoluble residue was washed with 50 mL of deionized water and then placed in a crucible. The crucible and the paper fibreglass were dried at 100 °C during 24 h to determine by weighting the amount of **Klason lignin**. After centrifugation of the sample in 2 mL Eppendorf® tubes , followed by filtration at 0.2-µm (Nylon membrane, Acrodlsc ®), 800 µL of supernatant were transferred to a vial prior to the analysis by high-pressure liquid chromatography (HPLC). Structural carbohydrates were measured by High Performance Liquid Chromatography (HPLC)

coupled to refractometric detection (Waters R410). The components were separated by an Aminex column HPX-87H column (300 × 7.8 mm, Biorad) equipped with a protective precolumn (Microguard cation H refill cartbridges, Bio-Rad). The eluting solution corresponded to 0.005 M H_2SO_4, and the flow rate was 0.3 mL min^{-1}. The column temperature was maintained at 50 °C and the refractometric temperature was fixed at 45°C. A refractive index detector (Waters 2414) was used to quantify the carbohydrates. The system was calibrated with glucose (0-6g L^{-1}), xylose (0-6g L^{-1}), arabinose (0-2g L^{-1}), and uronic acids (0-2g L^{-1}) (galacturonic and glucuronic) standards (Sigma–Aldrich®). Thereafter, cellulose and hemicelluloses contents were estimated as follows (equation II.1 and II.2):

Cellulose (% TS) = Glucose (%TS) / 1.11 (Equation II.1)

Hemicelluloses (% TS) = [Xylose (%TS) + Arabinose (%TS)] / 1.13 (Equation II.2)

where 1.11 is the ratio of the molecular weights of glucose to glucan (180/162) as (1/0.9) and 1.13 is the ratio of the molecular weights of xylose and arabionose to xylan (150/132) as (1/0.88).

4.3 Determination of the proteins content

The proteins content was estimated by multiplying the total Kjeldahl nitrogen by a factor of 6.25 (Izhaki, 1993). In this method, the total Kjeldahl 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 remaining boric acid was then titrated with an acid solution of 0.02 N HCl. The quantity of reacting boric acid corresponded to the total Kjeldahl nitrogen amount of the sample.

4.4 Determination of metabolites, residual sugars and byproducts of degradation

Volatile fatty acids (VFA) metabolites were quantified using a gas chromatograph (GC-3900, Varian). The liquid samples were collected in 2 mL Eppendorf® tubes and centrifuged at 5000 g during 10 min using a centrifuge (Eppendorf, Mini spin). 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) were added. VFA composition of the liquid phase, i.e. acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids were determined using a gas chromatograph (GC-3900, Varian) equipped with a flame ionization detector (FID).

Concentrations of non-VFA metabolic end products (lactate and ethanol), residual sugar monomers (glucose and xylose) and hydrolyzate byproducts (furfural and 5-hydroxylmethylfurfural) present in liquid phase were measured by High Performance Liquid Chromatography (HPLC) coupled to refractometric detection (Waters R410). The analytical chain was composed of an automatic sampler (Water 717plus), a pumping system (DIONEX UltiMate 3000), an oven (DIONEX ultimate 3000RS). After centrifugation of the samples in 2 mL Eppendorf® tubes, followed by filtration at 0.2 µm (Nylon membrane, Acrodlsc ®), 800 µL of supernatant were transferred to a vial prior to the analysis by high-pressure liquid chromatography (HPLC). The components were separated by an Aminex column HPX-87H column (300 × 7.8 mm, Biorad) equipped with a protective precolumn (Microguard cation H refill cartbridges, Bio-Rad). The eluting solution corresponded to 0.005 M H_2SO_4, and the flow rate was 0.4 mL min^{-1}. The column temperature was maintained at 35°C and the refractometric temperature was fixed at 45°C. Total phenols in the liquid fraction of the pretreated samples were determined using microtube test (Spectroquant®, Merck) followed by a colorimetric measurement method at 500 nm.

4.5 Biochemical changes and crystallinity measurement assessment by FT-IR

Fourier Transform Infrared Spectroscopy (FT-IR) spectroscopy was used to visualize the chemical composition changes induced by thermo-chemical pretreatments and to determine the crystallinity of lignocellulosic substrates. FT-IR spectra were collected in the 4000–600 cm^{-1} range using a Nexus 5700 spectrometer (ThermoElectron Corp.) with built-in diamond ATR single reflection crystal and with a cooled

MCT detector. Spectra were recorded in absorption mode at 4 cm^{-1} intervals with 64 scans, at room temperature. Three spectra were recorded for each sample and all spectra pre-treatments were analyzed using Omnic v7.3.software. The peaks of FT-IR spectra were assigned as follows: 1610 cm^{-1} to holocelluloses; 1512 cm^{-1} is characteristic of aromatic skeletal vibration C=C of lignin; 1430 cm^{-1} assigned to C-H deformation in cellulose; 1375 cm^{-1} assigned to deformation in cellulose and hemicelluloses; 1160 cm^{-1} assigned to C-O-C vibration in holocelluloses and 897 cm^{-1} to C-H deformation in cellulose (Shafiei et al., 2010; Yang et al., 2009). H Lignin / H carbohydrates ratio which shows the relative intensity of lignin peaks at 1512 cm^{-1} as opposed to carbohydrates peaks at 1610, 1430, 1375, 1160 and 897 cm^{-1} was calculated for untreated and thermo-chemically pretreated sunflower stalks in the chapter IV.

The bands ratio H 1430/ H 897 commonly called Lateral Order Indice (LOI) can be used to determine the amount of crystalline cellulose. Indeed, the bands at 1430 and 897 cm^{-1} are sensitive to the amount of crystalline cellulose and amorphous cellulose respectively (Spiridon et al., 2010). Using equations II.3 and II.4, the crystalline cellulose content was estimated (equation II.5).

Cellulose = Crystalline cellulose + Amorphous cellulose (Equation II.3)

LOI = Crystalline cellulose / Amorphous cellulose (Equation II.4)

Crystalline cellulose (IR) = Cellulose x CrI_{IR} (Equation II.5)

Where CrI_{IR} = LOI / (1+LOI)

4.6 Crystallinity by DRX

X-ray measurements were performed in a Philips Analytical X-diffractometer, using Cu Ka radiation at k = 0.1540 nm (40 kV, 40 mA). The measurements were carried out on powder compacted to small mats. DRX data were collected at 2θ angle range from 5° to 50° with a step interval of 0.02°. The degree of crystallinity was expressed as a percentage of crystallinity index (% CrI). The equation used to calculate the CrI was previously described by Segal et al. (1959) in the following form:

$CrI_{DRX} = (I_{002}-I_{am})/I_{002})*100$ (Equation II.6)

where I_{002} corresponds to the counter reading at peak intensity at a 2 θ angle of 22° and I_{am} the counter reading at peak intensity at 2θ angle of 16° in cellulose. I_{002} - I_{am} corresponds to the intensity of the crystalline peak and I_{002} is the total intensity after subtraction of the background signal measured without cellulose (Park et al., 2010). Crystalline cellulose was the determined using the equation II.7:

Crystalline cellulose (DRX) = Cellulose x CrI_{DRX} (Equation II.7)

4.7 Accessible surface and pore volume

Surface area and pore volume were evaluated by the BET technique according the method defined by Brunauer et al. (1993) method. Analysis were performed in the laboratory of "INANOTECH" (Rabat)

5. Characterization of the microbial communities

5.1 DNA extraction and PCR amplification

Liquid samples of 2 mL were collected from dark H_2 fermentation batch when the cumulative H_2 production was maximal and were centrifuged at 5000 g for 10 min in 2 mL Eppendorf® tubes. Samples were further conserved separately at -20 ˚C for microbial community analysis. DNA was extracted as described by Godon et al (1997). The amount and purity of DNA in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan).

To analyze the structure of the bacterial communities, the V3 region of the 16S rRNA genes were amplified using the bacterial primers 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˚C, followed by 25 cycles of 94˚C for 30s, 57˚C for 30s, and 72˚C for 30s, and a final extension at 72˚C

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. The amplified DNA samples were conserved at -20 °C prior to CE-SSCP analysis.

5.2 CE-SSCP electrophoresis

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. One µL of the appropriate dilution of the PCR amplified product was mixed with 18.8 µL of formamide and 0.2 µL of internal standard GeneScan ROX 400HD (Applied Biosystems). Samples were heat-denatured at 95°C for 10 min and immediately cooled in ice. Capillary Electrophoresis-Single Strand Conformation Polymorphism (CE-SSCP) was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) with 50 cm-long capillary tubes filled with a non-denaturing 5.6% conformation analysis polymer (Applied Biosystems). Samples were eluted at 12 kV and 32°C for 30 min (Hitachi Applied Biosystem 3130 Genetic Analyser).

Raw CE-SSCP data were analyzed using GeneScan software (Applied Biosystems). The CE-SSCP fingerprinting profiles were aligned with the internal standard ROX 400HD to take into account inter-sample electrophoretic variability. The CE-SSCP fingerprinting profiles were normalized using the StatFingerprints library (Michelland et al., 2009) from R software version 2.10.1 (R Development CoreTeam, 2009) according to standard procedure (Fromin et al., 2002). Each unique 16S rDNA sequence in a CE-SSCP fingerprinting profile gave a one peak. The area under a peak represented the relative abundance of the corresponding microbial species in the community.

6. Modeling

6.1 Hydrogen performance modeling of the BHP test

To determine the hydrogen production kinetic parameters, the cumulative H_2 production (H) data was fitted to a modified Gompertz equation (Equation II.8)

$$H(t) = P \exp\{-\exp[\frac{Rm.e}{P}(\lambda - t) + 1]\} \quad \text{(Equation II.8)}$$

where P is the maximum cumulative H_2 production (mol H_2 $mol^{-1}_{glucose}$ or mL H_2 g^{-1} VS), Rm is the maximum H_2 production rate (mol H_2 $mol^{-1}_{glucose}$ day^{-1} or mL H_2 g^{-1} VS day^{-1}), λ is the lag-phase time (days), t is the incubation time (days) and e is exp (1). The values of P, Rm and λ were estimated using a non-linear regression algorithm developed with Matlab software (version 6.5, MathWorks). The cumulative H_2 production was expressed in mol H_2 $mol^{-1}_{glucose}$ or mL H_2 g^{-1} VS taking into account the variations in volume due to gas and liquid sampling.

6.2 Methane performance modeling of the BMP test

The anaerobic digestion process was assumed to follow a first order kinetic as it is the case of substrate where hydrolysis is the limiting steps such as lignocellulosic residue (Angelidaki et al., 2009). Thus, to quantify the kinetic advantage of the pre-treatment on anaerobic methane production, the first order kinetic constants were calculated by using least-squares fit of methane production data during time (t) to the following equation:

$$B(t) = Bo\,(1-e^{-kt}) \qquad \text{(Equation II. 9)}$$

where B (t) is the volume of methane produced at time t (d), expressed in mL $CH_4.g^{-1}$ VS, Bo is the maximum producible methane volume (mL $CH_4.g^{-1}$ VS) and k is the hydrolysis kinetics constant (d^{-1}). Bo and k were determined using Microsoft Excel's Solver function.

6.3 Multivariable analysis of the data using Partial Least Square (PLS) regression

PLS (Partial Least Square) models were developed by using Unscrambler Version 10.2 software (CAMO software, A/S, Oslo, Norway). This method is particularly adapted for data with highly correlated variables. PLS models were used in full cross validation so-called leave-one-out cross validation procedure. This is a model validation method in which one sample is left out iteratively and a calibration model is built, and then the sample that was left out is predicted using this model (Raju et al., 2012). The iteration is continued until all samples are left once out of the calibration set. The prediction performances of the models were evaluated by the coefficient of determination (R^2) and the root mean square error of the calibration data set (RMSEPc). High R^2 and low RMSEPc values indicate a good predictive robustness of the model. PLS models built were

then tested on the validation independent set, the root mean square error of independent validation set (RMSEPiv) was calculated to define the quality of the model. The RMSEP was defined as follow:

$$RMSEP = \sqrt{\frac{\sum_1^n (\hat{y}i - yi)^2}{n}} \quad \text{(Equation II.10)}$$

where: $\hat{y}i$ is the prediction value of the sample i in a calibration data set (or independent validation set); yi, is the measured BHP or BMP value of the sample i in a calibration data set (or in a independent validation set) and n is the number of samples in calibration data set (or independent validation set). The uncertainty limit of the established model was calculated by multypling the RMSEPc by 1.96 as described by Lesteur et al. (2011)

6.4 Statistical evaluation using Anova

The analysis of variance (Anova) method was performed using Excel software (Microsoft) to analyze the impact of pretreatment parameters (time, temperature and acid concentration) on the methane potentials of the chapter VI.3. The confidence level considered was 95 % and statistical significance was recognized for $p < 0.05$.

7. Energy requirement

The heat energy requirement (HER) to treat 1 ton of TS of sunflower stalks during thermal-alkaline pretreatment from 25 °C to 55 °C was evaluated according the equation II-11.

$$\text{HER} = \left\{\frac{[m \times Cp^*(T\text{final} - T\text{initial})]}{3600}\right\} \quad \text{(Equation II-11)}$$

where HER is the heat energy requirement expressed in kWh t^{-1} TS, m is the mass of water and substrate in kg; Cp the water specific heat (4.18 kJ $kg^{-1}°C^{-1}$); T initial (°C) is the initial temperature of the substrate suspension, assumed as 25°C; T final (° C) is the final temperature of the substrate suspension.

Chapter III. Impact of compositional and structural features on biohydrogen and methane production from lignocellulosic residues

Adapted from "Monlau et al., 2012, Predictive models of biohydrogen and biomethane production based on the compositional and structural features of lignocellulosic materials, Environemental Science and Technolog, 46(21), 12217-12225.

1. Introduction

Lignocellulosic substrates are composed of three main fractions: lignin, cellulose and hemicelluloses. Contrary to the lignin compounds, the holocelluloses, ie cellulose and hemicelluloses, can be converted into biohydrogen and methane (Taherzadeh and Karimi, 2008). Additionally, lignocellulosic substrates present also structural features that limit the microbial accessibility to holocelluloses and thus their conversion to biohydrogen or methane.

Only few studies have attempted to give some insights on the effect of compositional and structural features of lignocellulosic substrates on biohydrogen and methane production (Guo et al., 2012, Gunasselan et al., 2009; Gunaseelan et al., 2004, Buffière et al. 2006; Triolo et al., 2011). The correlations found in the literature between the compositional features and the associated biohydrogen or methane productions from lignocellulosic residues are summarized in Table III.1.

Table III. 1 *Correlations found in the literature between the compositional characteristics of lignocellulosic substrates and biohydrogen or methane production.*

Fermentation processes	Biomass used	Compositional features	Equation	References
Biohydrogen	organic solid substrates (n=21)	Soluble Carbohydrates (Carb)	BHP (mL H_2 g^{-1} TS) = 1.31 + 199.46 Carb ($g.g^{-1}$TS)	Guo et al., 2012
Methane	Manure (n=10), Energy crops (n=10)	Lignin (Lig)	BMP (L CH_4 kg^{-1} VS) = 421.7 - 1.67*Lig (g kg^{-1}VS)	Triolo et al., 2011
Methane	Raw and thermo-chemically pretreated sunflower stalks (n= 8)	Lignin (Lig)	BMP (L CH_4 kg^{-1}VS) = 379.8 - 0.65*Lig (g g^{-1}VS)	Monlau et al., 2012a
Methane	Lignocellulosic residues (n=7)	Soluble carbohydrates (Carb), acid detergent fiber (ADF), Protein (Pro), Lignin (Lig), Ash (A)	BMP (L CH_4 kg^{-1} VS) = 0.18 + 0.48 *Carb + 0.2 * ADF -0.003 * Lig/ADF + 2.8 Pro - 0.83 *A (g g^{-1}VS)	Gunaseelan et al., 2007
Methane	Lignocellulosic residues (n=12)	Soluble carbohydrates (Carb), acid detergent fiber (ADF), Protein (N), Ash (A), Lipids (F)	BMP (L CH_4 kg^{-1} VS) = 0.045 + 1.23* Carb + 0.24 * Pro + 1.51* F -0.68 * ADF -0.81* Cell - 6.1*A (g g^{-1}VS)	Gunaseelan et al., 2009
Methane	Lignocellulosic residues (n=15)	Lignin (Lig)	Biodegradability (%MV) = 0.83-1.82*Lig	Chandler et al., 1980
Methane	Municipal solid waste (n=2), agricultural residues (n=2), manure (n=4), vegetables (n=6)	Lignin (Lig), cellulose (Cell)	Biodegradability (%DCO) = 0.87-1.03 (lignin + cellulose) ($g.g^{-1}$VS)	Buffiere et al., 2006

Except the models established by Gunasselan et al. (2007 and 2009), all models previously described were built with one or two compositional characteristics. Moreover, only compositional features were considered and structural characteristics such as crystallinity of cellulose have not been yet considered. Moreover, other compositional characteristics such as the presence of pectin (polymer of uronic acids) have not been investigated in these models. Recently, Pakarinen et al. (2012b) showed that the removal of pectin can increase significantly the enzymatic hydrolysis of lignocellulosic substrates.

Information about the influence of compositional and structural features on fermentative processes is thus still poor especially for hydrogen production and sometimes results are contradictory. Thus, the main objective of this chapter was to investigate the effect of compositional and structural features (lignin (Lig), crystalline cellulose (Cri), amorphous holocelluloses (Am), soluble carbohydrates (SolSu), proteins (Pro), total uronic acids (sum of glucuronic and galacturonic acids, Ua) on biohydrogen and methane from lignocellulosic residues.

To achieve this objective, structural and compositional features previously defined were determined for twenty lignocellulosic residues. Their biohydrogen and methane potentials were determined through batch tests. Structural and compositional features were further correlated to the methane and biohydrogen production through Partial Least Square regression models (PLS).

2. Correlation between crystalline cellulose determined by FT-IR and DRX

To validate the use of FT-IR spectra to evaluate crystallinity, crystallinity was also determined by a more common technology, as X-ray diffraction, on eight lignocellulosic substrates (giant reed stalks, sunflower stalks NK-Kondi, maize stalks, rice straw, sorghum 1, Jerusalem artichoke stalks, maize cobs and sunflower oil cakes). Even though the crystalline cellulose determined by FT-IR was higher than DRX, a good correlation (R^2 = 0.93) was found between the crystalline cellulose amounts determined by DRX and FT-IR (Figure III.1). Similar linear correlation was previously observed by Marson and El Seoud (1999) between indices of crystallinity determined by FT-IR

spectroscopy and DRX. Consequently, FT-IR can be considered as an appropriate method to compare crystalline cellulose content in lignocellulosic substrates.

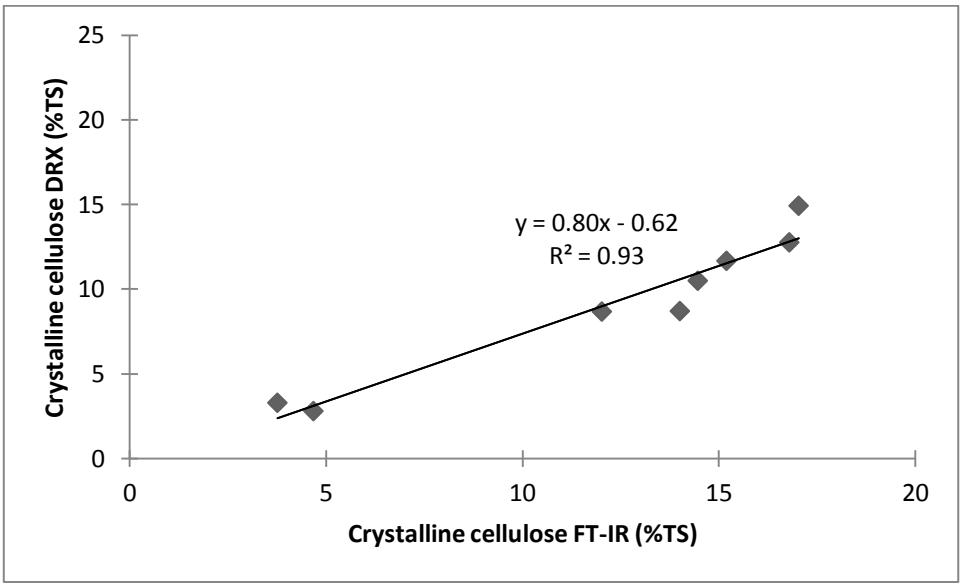

Figure III. 1 *Correlation between crystalline celluloses determined by FT-IR and DRX (expressed in % TS).*

3. Compositional and structural features of lignocellulosic substrates

Soluble sugars (SolSu), uronic acids (Ua), proteins (Pro), hemicelluloses (Hem), cellulose (Cell), and lignin (Lig) contents of twenty lignocellulosic substrates are presented in Table III.2, in % of TS. Soluble sugars (starch, sucrose and inulin) were mainly present in sorghum substrates (ranging from 8.2 to 22.8 %, except for sorghum 1). Gunaseelan et al., (2007) noticed also a high content of soluble carbohydrates up to 23 % of VS in sorghum bicolour roots (Gunaseelan et al., 2007). According to Thuesombat et al. (2007), Jerusalem artichoke presents 70-90% of inulin (linear poly-fructose chain) which explains the high values of soluble sugars found for Jerusalem artichoke stalks and tubers, ie 32.9 % and 59.1 % TS respectively. Protein content ranged from 2.3 % (sunflower stalk 2) to 29.7 % (sunflower oil cakes). This result is consistent with Raposo et al., (2008) who evaluated a protein content of 31 % TS in sunflower oil cakes. Uronic acids (galacturonic and glucuronic) which originated from both pectins and hemicelluloses were also

quantified. Uronic acids contents ranged from 0.2 % (giant reed and Jerusalem artichoke stalks) to 7 % (sunflower stalks 1). Concerning the holocelluloses fraction, hemicelluloses content ranged from 5 % (Jerusalem artichoke tubers) to 34.6 % (maize cobs) and cellulose contents ranged from 5.4 % (Jerusalem artichoke bulbs) to 33.1 % (giant reed stalks). Crystalline cellulose and amorphous holocelluloses, expressed in % TS using FTIR spectra, are presented in Table III.2. The crystalline cellulose content ranged from 2.5 % for Jerusalem artichoke bulbs to 16.3 % for giant reed stalks. The content of amorphous holocelluloses, which is the sum of amorphous cellulose and hemicelluloses, ranged from 7.5 % TS (Jerusalem artichoke tubers) to 50.3 % TS (maize cobs). Finally, lignin content ranged from 12.3 % (Jerusalem artichoke tubers) to 35 % (sunflower stalks bark). Moreover, by comparing different parts of a same plant, lignin content was found higher in stalks than in leaves, except for giant reeds that presented almost similar lignin content in leaves and stalks. Similar trends were observed by Fukushima et al. (2004) with 14.1 % and 18.4 % of lignin for wheat straw leaves and stalks, respectively. The range of the variables values (%TS) investigated was relatively high to permit to screen a wide range of compositional and structural features (Table III.2).

Table III. 2 *Compositional and structural features of lignocellulosic substrates and validity range of PLS models. Except for soluble sugars contents, values correspond to the means of two replicates of independent values ± standard deviations (error bars).*

		Chemical composition (% TS)						FT-IR spectra	
Substrates	**% TS**	**SolSu**	**Pro**	**Ua**	**Hem**	**Cell**	**Lig**	**LOI**	**CrI (% TS)**	**Am (% TS)**
Rice straw	0.96	0.8	5.3 (± 0.2)	0.6 (± 0.1)	18.8 (± 0.7)	26.2 (± 0.5)	27 (± 2.6)	0.85	12	33
Giant reed stalks	0.99	0.3	4.3 (± 0.7)	0.2 (± 0.0)	18.5 (± 0.7)	33.1 (± 1.3)	24.5 (± 0.1)	1.03	16.8	34.8
Giant reed leaves	0.99	2.9	8 (± 0.1)	0.7 (± 0.1)	17.7 (± 0.6)	20.9 (± 0.6)	25.4 (± 0.1)	0.95	10.2	28.4
Sunflower stalks 1	0.94	0.0	4.8 (± 0.1)	7.0 (± 0.6)	15.6 (± 0.3)	31 (± 1.6)	29.2 (± 1.6)	1.22	17	29.6
Sunflower stalks 2	0.96	0.0	2.3 (± 0.4)	3.9 (± 0.4)	14.3 (± 2.4)	31.2 (± 3.1)	27.7 (± 0.2)	1.20	17	28.4
Sunflower stalks 3	0.96	0.0	4.3 (± 0.7)	2.4 (± 0.2)	14.3 (± 0.7)	31.3 (± 0.7)	30 (± 1.7)	1.18	16.9	28.6
Sunflower stalks bark	0.97	0.0	2.8 (± 0.4)	1.7 (± 0.3)	13.5 (± 0.2)	27.4 (± 0.4)	35 (± 0.4)	1.10	14.4	26.5
Sunflower oil cakes	0.94	5.2	29.7 (± 3.4)	1.4 (± 0.2)	8.2 (± 0.2)	5.1 (± 0.3)	22.3 (± 2.8)	0.96	3.8	12.1
Maize stalks	0.99	0.4	7.4 (± 0.1)	0.7 (± 0.1)	21.2 (± 0.6)	27.1 (± 0.9)	23.2 (± 0.1)	1.14	14.5	33.9
Maize leaves	0.99	0.3	6.7 (± 0.6)	1.0 (± 0.2)	28.6 (± 3.3)	30.9 (± 3.1)	20.4 (± 0.6)	1.03	15.7	43.8
Maize cobs	0.96	0.2	4.3 (± 0.3)	0.7 (± 0.1)	34.6 (± 1.4)	29.8 (± 1.2)	19.2 (± 1.0)	0.89	14	50.3
Jerusalem artichoke stalks	0.96	32.9	2.8 (± 0.3)	0.2 (± 0.0)	8.8 (± 3.1)	9.6 (± 3.1)	20.3 (± 0.0)	0.95	4.7	13.7
Jerusalem artichoke leaves	0.94	2.6	12.4 (± 0.3)	0.7 (± 0.1)	4.7 (± 0.6)	8.8 (± 1.4)	12.9 (± 1.3)	1.22	4.8	8.6
Jerusalem artichoke tubers	0.98	59.1	10.4 (± 0.2)	1.5 (± 0.2)	5 (± 0.0)	5.4 (± 0.3)	12.3 (± 0.1)	1.10	2.8	7.5
Sorghum 1	0.95	0.4	4.6 (± 0.1)	0.9 (± 0.1)	26.1 (± 0.1)	29.1 (± 0.3)	22.5 (± 1.6)	1.09	15.2	40
Sorghum 2	0.91	15.4	6.5 (± 0.0)	0.6 (± 0.1)	19.4 (± 1.3)	22.2 (± 1.5)	21.4 (± 0.3)	1.05	11.4	28.3
Sorghum 3	0.91	18.5	8.1 (± 0.0)	1.0 (± 0.0)	20.9 (± 1.6)	20.1 (± 1.7)	18.5 (± 0.9)	0.98	10.3	27.9
Sorghum 4	0.94	8.2	8.2 (± 0.0)	0.6 (± 0.0)	21.7 (± 0.2)	18.3 (± 5.8)	20.7 (± 3.0)	1.03	9.3	27.7
Sorghum 5	0.92	22.8	6.9 (± 0.0)	0.6 (± 0.0)	20 (± 1.2)	19.7 (± 0.2)	19.8 (± 1.3)	1.11	10.4	26.2
Sorghum 6	0.88	21.3	6.2 (± 0.0)	0.6 (± 0.0)	18.5 (± 0.8)	18.1 (± 0.1)	21.3 (± 0.0)	1.10	9.5	24.4
Validity range		*0-59.1*	*2.3-29.7*	*0.2-7*	*4.7-34.6*	*5.4-33.1*	*12.3-35*		*2.5-16.3*	*7.5-50.3*

4. Biohydrogen and methane production from lignocellulosic residues

Hydrogen and methane potentials of lignocellulosic substrates expressed in mL H_2 g^{-1}TS and mL CH_4 g^{-1}TS respectively are presented in Figure III.2.

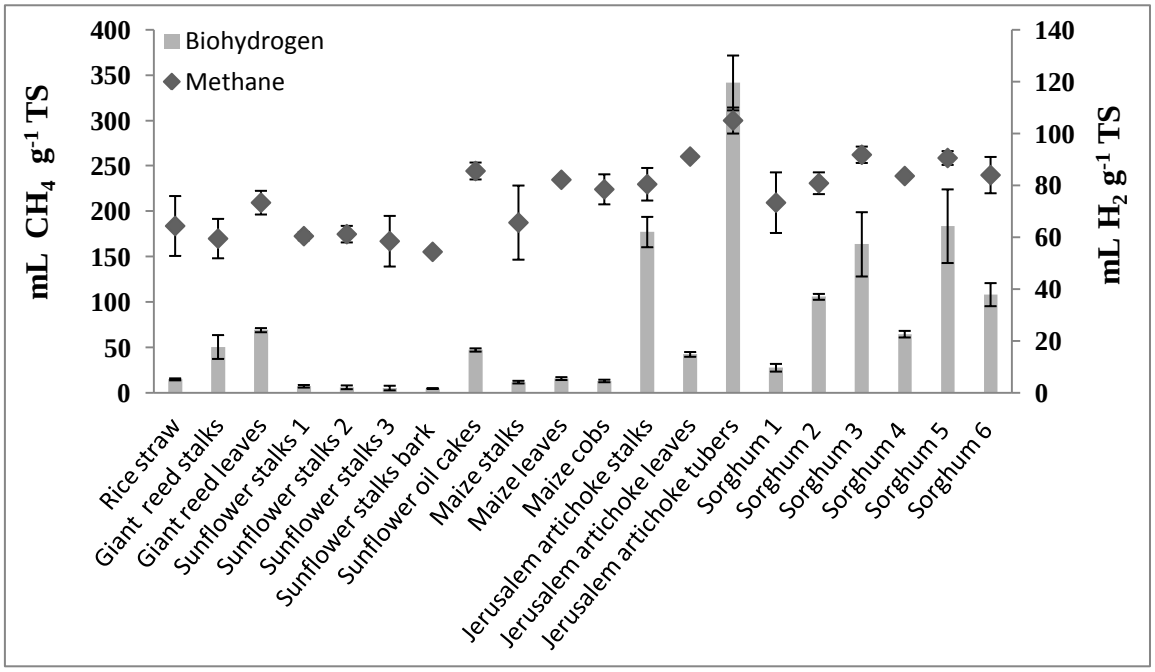

Figure III. 2 *Biochemical biohydrogen and methane potentials of lignocellulosic substrates. Values correspond to the means of two replicates of independent values ± standard deviations (error bars).*

Hydrogen potential ranged from 1.6 (± 0.1) mL H_2 g^{-1}TS (sunflower stalk bark) to 120 (± 11) mL H_2 g^{-1}TS (Jerusalem artichoke tubers). Similarly to Jerusalem artichoke tubers, Jerusalem artichoke stalks, which produced 62 (± 6) mL H_2 g^{-1} TS, have an interesting bio-hydrogen potential. Except for sorghum 1, high sorghum hydrogen potentials were observed for sorghum substrates (from 23 (± 1) mL H_2 g^{-1} TS to 64 (± 14) mL H_2 g^{-1} TS). Similar hydrogen yields were reported on sweet sorghum stalks with 52 mL H_2 g^{-1} VS (Shi et al., 2010). Prakasham et al. (2012) found also similar hydrogen potentials from 59 to 72 mL H_2 g^{-1} TS on three sorghum derivatives from sweet sorghum. Sunflower stalks were found to produce low hydrogen potentials (1.8 (± 0.9), 2.1 (± 0.7) and 2.5 (± 0.5) mL H_2 g^{-1} TS for sunflower stalks numbers 3, 2, and 1, respectively). Among similar lignocellulosic residues, lower hydrogen yields (1 mL H_2 g^{-1} VS and 3.16 mL H_2 g^{-1} VS) were observed for wheat straw and cornstalks, respectively

(Fan et al., 2005; Zhang et al., 2006). In addition, methane potentials ranged from 155 (± 2) mL CH_4 g^{-1} TS (sunflower stalks bark) to 300 (± 14) mL CH_4 g^{-1} TS (Jerusalem artichoke tubers). Such results are also in agreement with literature data as Dinuccio et al. (2010) found methane potentials of 317 mL CH_4 g^{-1}VS for maize residues, 229 mL CH_4 g^{-1}VS for barley straw, and 195 mL CH_4 g^{-1} VS for rice straw. Besides Jerusalem artichoke tubers, interesting methane production of 230 (± 18) and 260 (± 4) mL CH_4 g^{-1} TS were respectively observed for Jerusalem artichoke stalks and leaves, respectively. All sorghum substrates present methane potentials higher than 210 (± 33) mL CH_4 g^{-1} TS. Maize leaves and sunflower oil cakes also led to good methane potentials with respectively 235 (± 3) and 244 (± 9) mL CH_4 g^{-1}TS. Low methane potentials were observed for the different varieties of sunflower stalks as 167 (± 27), 172 (± 5), and 175 (± 9) mL CH_4 g^{-1}TS for sunflower stalks numbers 3, 1, and 2, respectively. Moreover, on a same plant, the leaves appeared to have higher methane potentials than stalks. As an example, methane potentials of 170 (± 22) and 210 (± 13) mL CH_4 g^{-1} TS were respectively observed for giant reed stalks and leaves, respectively. Overall, all results kept lower than 480 mL CH_4 kg^{-1} TS which is the theoretical methane potential of lignocellulosic substrates determined by Frigon and Guiot (2010). Some biodegradable parts are indeed not accessible during anaerobic digestion of lignocellulosic substrates, likely due to the compositional and structural characteristics that limit the accessibility of microorganisms to holocelluloses, as previously suggested by Triolo et al. (2011).

4.1 Impact of compositional and structural features on fermentative processes

One of the main objectives of this chapter was to identify the compositional and structural features affecting both hydrogen and methane production from lignocellulosic residues, such as lignin (Lig), amorphous holocelluloses (Am), crystalline cellulose (Cri), protein (Pro), uronic acids (Ua) and soluble sugars (SolSu) contents. PLS models were built on eighty lignocellulosic substrates and an independent validation set of two substrates (sorghum 1 and sorghum 6) was used to validate the PLS models built. Table III.2 shows the range values of the variables (Lig, Am, Cri, Pro, Ua, SolSu) in which PLS models are relevant and should not be extrapolated out of these ranges.

4.2 Impact on biohydrogen production

PLS analysis led to equation III-1 as by a multi-linear model for biohydrogen potentials. The quality of the model to predict hydrogen potential was confirmed by a good R^2 (0.87) and a low value of RMSEPc (11.6 mL H_2 g^{-1} TS).

BHP (mL H_2 g^{-1} TS) = 19.43 + 1.84* SolSu (g g^{-1} TS) – 0.36* Lig (g g^{-1} TS) + 0.53Ua (g g^{-1} TS) - 0.14*Cri (g g^{-1} TS) - 0.05Am (g g^{-1} TS) - 0.02*Pro (g g^{-1} TS) (Equation III-1)

This model was validated using a set of two independent samples (sorghum 1 and sorghum 6) which were not included in the calibration data set. Results are presented in Table III.3. Hydrogen potentials of 9.2 and 45.9 mL H_2 g^{-1} TS were predicted compared to 9.7 and 37.9 mL H_2 g^{-1} TS measured respectively for sorghum 1 and 6. The REMSEPiv was calculated on the validation data set and promising results of 5.7 mL H_2 g^{-1} TS was observed showing the high accuracy of the model.

Table III. 3 *External validation of the PLS models for biohydrogen potentials.*

	PLS model for biohydrogen potentials			
Independent samples	**BHP measured mL H_2 g^{-1} TS**	**BHP predicted mL H_2 g^{-1} TS**	**Errors**	**RMSEPiv**
Sorghum 1	9.7	9.2	4.5%	5.7
Sorghum 6	37.9	45.9	21.1%	

Another interest of the PLS models is to determine variables which significantly impact the predicted variable. Centred and reduced weighted regression coefficients for hydrogen potentials are shown in Figure III.3a.

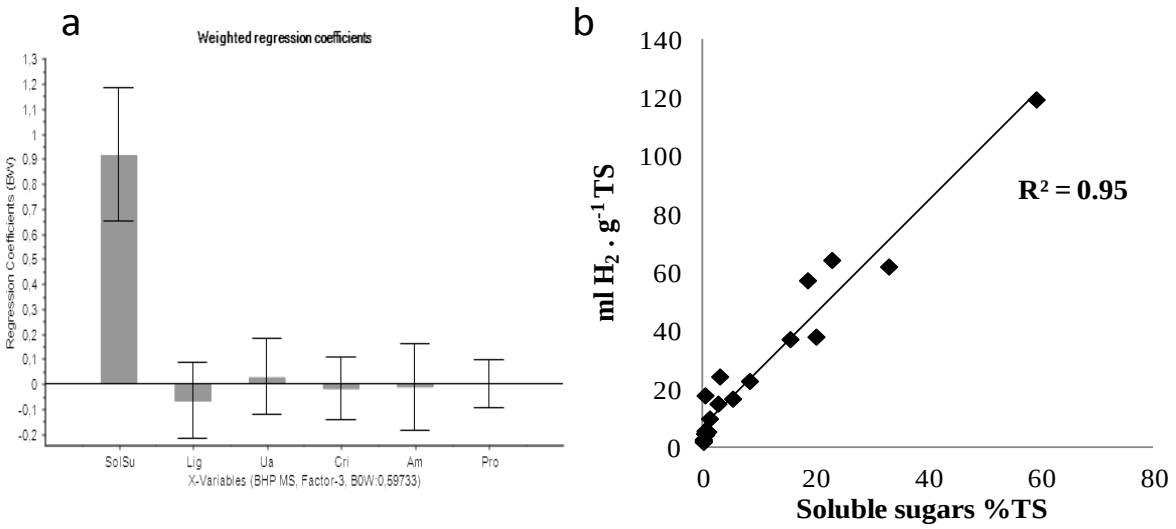

Figure III. 3 *Centred and reduced regression coefficients for the prediction of biohydrogen potentials (a) Correlation between hydrogen potentials and soluble sugars amounts of lignocellulosic substrates (b).*

A strong positive correlation was found between hydrogen potentials and soluble sugars (SolSu) whereas all other studied variables (Lig, Am, Cri, Ua, Pro) had no significant impact. Considering the correlation of hydrogen production versus only soluble carbohydrates, a high coefficient correlation of R^2 = 0.95 was observed (Figure III.3b). These results are in accordance with Zhang et al., (2006) who suggested that the hydrogen yield enhancement was concomitant to an increase of the soluble sugar content of the substrate. Recently, Guo et al. (2012) found a similar correlation (R^2 = 0.87) between biohydrogen potentials and carbohydrates contents, extracted under mild conditions (2 N hydrochloric acid). Accordingly Pan et al. (2011), showed that the hydrolysis of cellulosic biomass led to an enhancement of hydrogen production due to an increase of soluble substrates that were much easier to be degraded.

In addition, our results showed that protein content did not affect hydrogen potentials which is in agreement with Guo et al. (2012) who reported lower hydrogen potentials from protein rich-substrate than soluble carbohydrates rich-substrates. However, the pH is an important factor that can affect biohydrogen production. Although, the optimal pH for hydrogen production from carbohydrates is acidic (about 4.5 - 6), pH is more favourable for proteins rich substrates if alkaline (about 8.5-11) (Cai et al., 2004; Xiao et al., 2010). In our study, the initial pH was set up at 5.5 that can explain the absence

of significant effect of protein content. The absence of significant positive correlation between amorphous holocelluloses with hydrogen potentials can be explained by the poor efficiency on direct assimilation of cellulosic materials by H_2 producing bacteria (Saratale et al., 2008). To achieve high yield of hydrogen from lignocellulosic substrates, an hydrolysis step is required (Saratale et al., 2008).

4.3 Impact on methane production

PLS analysis led to equation III-2 as a multi-linear model for methane potentials. The quality of the model to predict methane potential was confirmed by a high R^2 (0.88) and a low value of RMSEPc (14.9 mL CH_4 g^{-1} TS).

BMP (mL CH_4 g^{-1} TS) = 303.14 – 4.53*Lig (g g^{-1} TS) + 0.77*SolSu (g g^{-1} TS) + 1.28*Pro (g g^{-1} TS) - 1.59*Cri (g g^{-1} TS) + 0.61Am (g g^{-1} TS) +1.33Ua (g g^{-1} TS) (Equation III-2)

This model was validated using a set of two independent samples (sorghum 1 and sorghum 6) which were not included in the calibration data set. Results are presented in Table III.4. Errors between experimental and predicted methane potentials of 0.2 % and 4 % were observed respectively for sorghum 1 and sorghum 6. The REMSEPiv was calculated on the validation data set and results of 6.7 mL CH_4 g^{-1} TS were observed showing the high accuracy of the models. Centred and reduced regression coefficients for the prediction of methane potentials are presented in Figure III.4a.

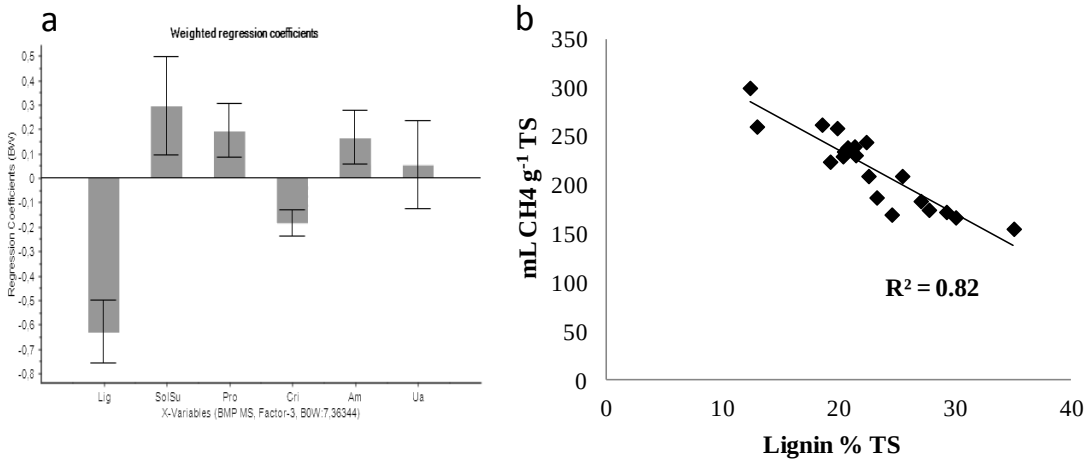

Figure III. 4 *Centred and reduced regression coefficients for the prediction of methane potentials (a) Correlations between methane potentials and lignin amounts of lignocellulosic substrates (b).*

In this case, lignin, crystalline cellulose, soluble sugars, amorphous holocelluloses and proteins contents were found to have a significant effect on methane potentials. A strong negative correlation was found between the lignin content and the methane production which is in agreement with other reported studies (Chandler et al., 1980; Monlau et al., 2012a, Kobayashi et al., 2004; Triolo et al, 2011). Kobayashi et al. (2004) showed a strong negative correlation ($R^2 = 0.95$) between the amount of methane produced and the amount of lignin of steam explosed bamboo. Triolo et al. (2011) also found a high negative correlation ($R^2 = 0.88$) between the lignin content and methane potentials of energy crops and manure. Nevertheless, our results led to a satisfactory correlation (R^2 of 0.82, Figure III.4b) between the lignin content and the methane potentials, showing that the anaerobic biodegradation of lignocellulosic materials into methane was not only related to the lignin content, as suggested by Triolo et al. (2011).

PLS regression showed that crystalline cellulose had a negative impact on methane production in a lower extent. Zhu et al. (2010b) showed that lignin content and crystallinity are the two dominant parameters affecting negatively the digestibility of lignocellulosic substrates. Moreover, they suggested that the crystallinity of cellulose had higher influence on short time hydrolysis, whereas the lignin content had higher influence for long time hydrolysis (Zhu et al., 2010b).

Additionally, significant positive correlation was found between the methane potentials and the contents in soluble sugars, proteins and amorphous holocelluloses in our study. According to Hayashi et al. (2005), the readily accessible regions (amorphous regions) of the lignocellulosic biomass are more efficiently hydrolyzed during enzymatic hydrolysis, resulting in the accumulation of crystalline cellulose (Hayashi et al., 2005). Similarly, Scherer et al. (2000) showed that the most degradable part of the organic matter of spent grains was the soluble part and hemicelluloses fraction, while cellulose and lignin were slightly degraded (Scherer et al., 2000).

Table III. 4 *External validation of the PLS models for methane potentials.*

	PLS model for methane potentials			
Independent samples	**BMP measured mL CH_4 g^{-1} TS**	**BMP predicted mL CH_4 g^{-1} TS**	**Errors**	**RMSEPiv**
Sorghum 1	209.5	209	0.2%	6.7
Sorghum 6	240	230.5	4.0%	

5. Conclusions

Biohydrogen and methane production from some lignocellulosic substrates is possible as hydrogen potential ranged from 1.6 mL H_2 g^{-1} TS (sunflower stalk bark) to 120 mL H_2 g^{-1} TS (Jerusalem artichoke tubers) and methane potentials ranged from 155 mL CH_4 g^{-1} TS (sunflower stalks bark) to 300 mL CH_4 g^{-1} TS (Jerusalem artichoke bulbs) were observed. Nevertheless, values are still lower than theoretical biohydrogen and methane potentials that can be expected if all biodegradable matter was converted suggesting that some compositional and structural features limit the lignocellulosic conversion into biohydrogen and methane. For biohydrogen conversion, only soluble sugars were found to have a significant positive effect on hydrogen production (Figure III.5). For methane conversion, a negative correlation was observed between methane potential and lignin content. In a lesser extent, crystalline cellulose content showed also a negative impact on methane potential. On the contrary, soluble sugars, proteins and amorphous holocelluloses were found to have a positive impact on the methane production (Figure III.5). Besides, giving a quick tool to predict biohydrogen and methane potentials from lignocellulosic substrates, PLS models previously build can be also valuable to give directions towards the development of pretreatments strategies of lignocellulosic residues for enhancing both biohydrogen and methane production. Pretreatments leading to the solubilisation of hollocelluloses may be recommended for biohydrogen production whereas delignification, hollocelluloses solubilisation and reducing crystalline cellulose may be recommended for methane production.

In the next chapter, different thermo-chemical and enzymatic pretreatments or combination of the two will be carried out to modify the compositional and structural features. Sunflower stalks which were the most recalcitrant substrates in term of biohydrogen and methane production were selected as the model substrate to performed pretreatments.

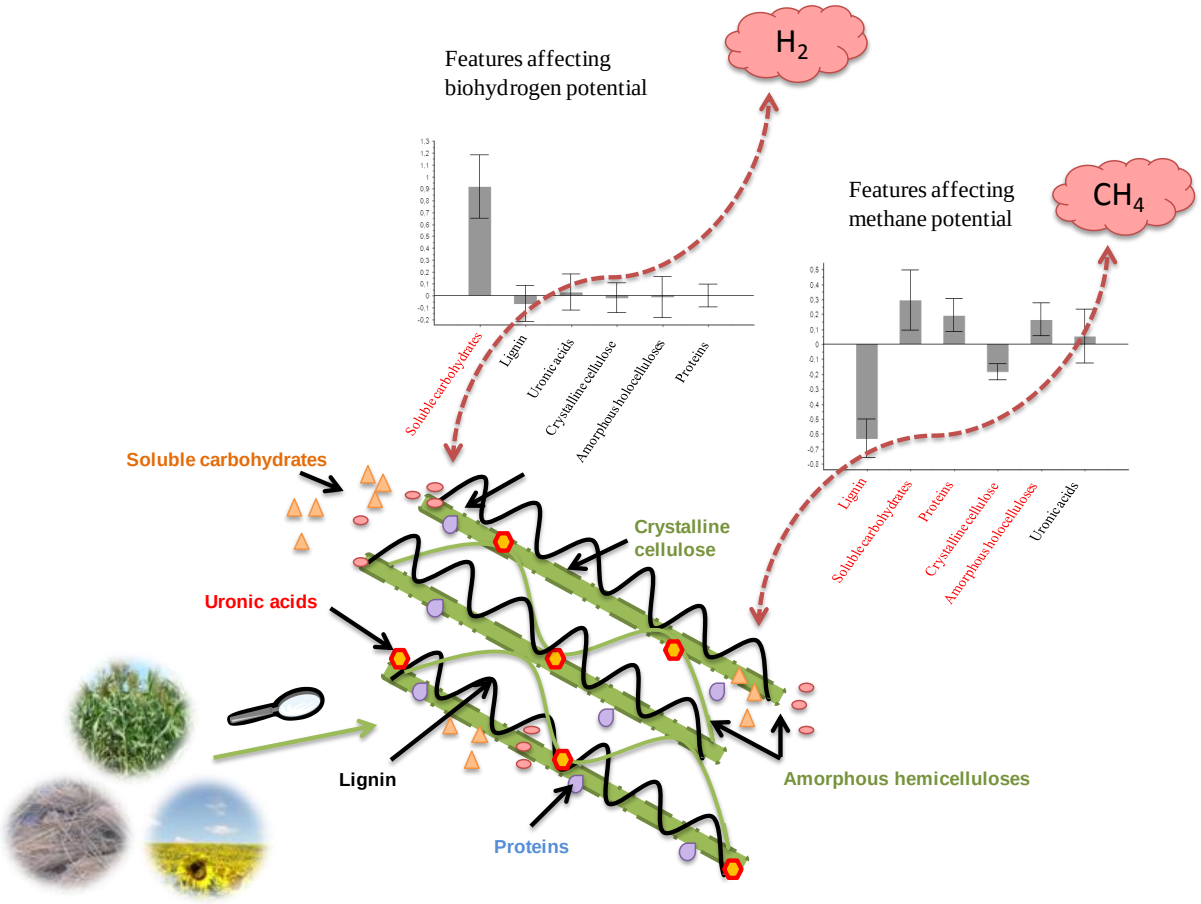

Figure III. 5 *Overall scheme of the compositional and structural features affecting both biohydrogen and methane production from lignocellulosic substrates.*

Chapter IV. Effect of thermo-chemical and enzymatic pretreatments on chemical composition and structural features of sunflower stalks.

Adapted from "Monlau et al., 2012, Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks, Bioresource Technology, 120, 241-247.

1. Introduction

The aim of this part was to study the effect of different pretreatments on chemical composition (lignin, cellulose, hemicelluloses, proteins, uronic acids) and structural features (crystallinity of cellulose) of lignocellulosic biomass. The ultimate goal was to choose the best pretreatments strategies that will answer to the objectives defined in the previous chapter: solubilization of carbohydrates for hydrogen production, removal of lignin, increase of carbohydrates contents and decrease of cellulose crystallinity for methane production. Thus, various thermo-chemical pretreatments, enzymatic pretreatments, and combination of both, have been investigated to modify the chemical composition and structural features of lignocellulosic substrates. Further, these pretreatments will be used to increase both biohydrogen and methane production. According to previous results (chapter III), the most recalcitrant substrate in term of biohydrogen and methane production (i.e. sunflower stalks) has been selected to apply various pretreatments (thermo-chemical and enzymatic).

Therefore, two thermal (55°C, 170°C) and five thermo-chemical pretreatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) were carried out on NK-Kondi sunflower stalks. Chemical agents were chosen according to literature data, as describe next.

As for acidic pretreatments (sulfuric acid, hydrochloric, phosphoric, maleic, acid or nitric acids), sulfuric acid is the most widely used (Fernandes et al., 2009; Taherzadeh and Karimi, 2008). Nevertheless, sulfuric acid pretreatments, contrary to other acids present the disadvantage to generate some sulfate ions SO_4^{2-} in the hydrolysate. Sulfate is a stronger electron acceptor than CO_2 and the reduction of sulfate to H_2S competes with the methane process (Zehnder and Stumm, 1988). On the contrary, inorganic salts, especially $FeCl_3$, are efficient in hemicelluloses removals and thus can be interesting for further enhancement of methane or hydrogen production (Liu et al., 2009 a,b). Moreover, the presence of trace element such as Fe in anaerobic digester can significantly improve the performance of anaerobic digestion process (Demirel and Scherer, 2011). Thus, HCl and $FeCl_3$ were selected.

As for alkaline and oxidative pretreatments (NaOH, $Ca(OH)_2$, H_2O_2), they are effective in increasing the accessible surface by removing part of the lignin and hemicelluloses (Taherzadeh and Karimi, 2008; Xie et al., 2011). Alkaline pretreatments have been investigated to enhance hydrogen or methane production from lignocellulosic substrates (Fernandes et al., 2009; Xie et al., 2011, de Vrije et al., 2002, Han et al., 2012). Alkaline pretreatment involves the use of bases such as sodium, potassium, calcium and ammonium hydroxide. Sun et al. (1995) studied the effectiveness of different alkaline solutions by analyzing the delignification and dissolution of hemicelluloses in wheat straw. They found that the optimal condition was using 1.5% sodium hydroxide for 144 hours at 20°C, which resulted in 60% release of lignin and 80% release of hemicelluloses. Among alkaline pretreatments, the advantage to use calcium hydroxide is its cheapest cost required to pretreat a given quantity of biomass. For example, in 2005, the estimated cost of materials was $70/ton hydrated lime compared to $270/ton fertilizer grade ammonia and $320/ton for 50 wt% NaOH and 45wt% KOH (Brodeur et al., 2011). Few studies have investigated oxidative pretreatments to enhance hydrogen and methane potentials maybe due to the fact that pH should be adjusted to 11.5. Indeed, hydrogen peroxide was found more efficient to delignify lignocellulosic substrate at an optimal pH of 11.5 (Gould, 1985). Thus among oxidative and alkaline pretreatments NaOH, $Ca(OH)_2$, H_2O_2 will be investigated.

Pretreatments conditions were chosen according to literature data, suggesting that acid pretreatments are used at high temperature (160-220°C) and short time period (several minutes or few hours), whereas alkaline or oxidative pretreaments are often carried out at low temperature over a long period (some hours or days) (Mosier et al., 2005; Taherzadeh and Karimi, 2008). Thus, alkaline and oxidative pretreatments (NaOH, H_2O_2, $Ca(OH)_2$) were performed at 55°C during 24 hours whereas acidic pretreatments (HCl and $FeCl_3$) were carried out at 170°C during 1 hour. Besides, thermo-chemical pretreatments, two thermal pretreatments were performed at 55°C and 170°C to study the impact of temperature without chemical reagent.

In a second part, an enzymatic pretreatment (cocktail of cellulase, xylanases and β-glucosidase) was investigated to solubilise cellulose and hemicelluloses of sunflower stalks. However, in the lignocellulosic

matrix, holocelluloses and lignin are strongly linked to each other and form complex three dimensional structures less available to enzymes (He et al., 2008). Indeed, accessibility of enzymes or microorganisms to holocelluloses in the lignocellulosic matrix is known to be very limited and alkaline pretreatment is known to remove efficiently lignin and thereafter to increase accessible surface area for further enzymatic or microbial attack (Taherzadeh and Karimi, 2008). Thus, the combination of alkaline and enzymatic pretreatments was also investigated.

2. Impact of thermo-chemical pretreatments

2.1 Impact on chemical composition

Results on the investigation of biochemical changes induced by thermo-chemical pretreatment on protein, uronic acids, hemicelluloses, cellulose and Klason lignin content of NK-Kondi sunflower stalks are presented in Figure IV.1. First, "NK-Kondi" sunflower stalks were composed of proteins, uronic acids, hemicelluloses, cellulose and lignin at, respectively, 5.2%, 7.6%, 20.8 %, 34 % and 29.7 %.

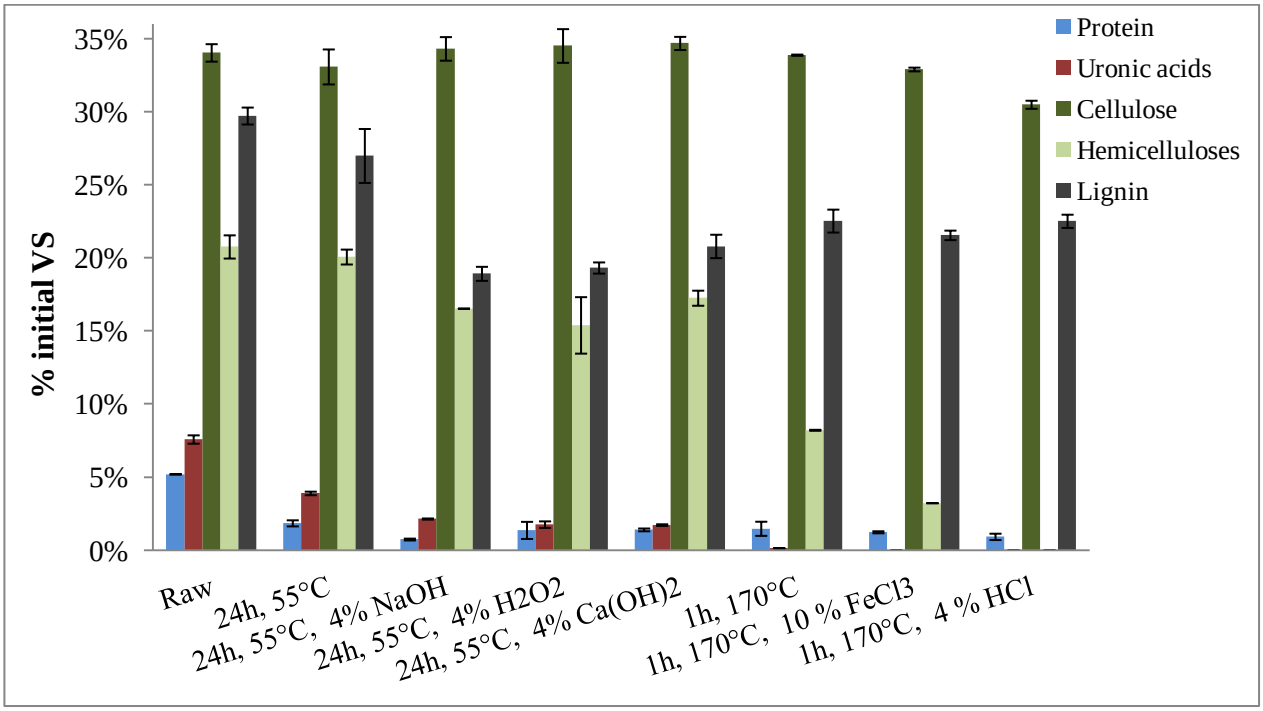

Figure IV. 1 *Biochemical composition of raw and of the solid residue after thermo-chemical pretreatments of sunflower stalks NK-Kondi. Values correspond to means of two replicates of independent values ± standard deviation (bar errors).*

All thermo-chemical pretreatments were effective in proteins removal; even at 55°C for 24 h, more than 60% of the proteins were removed from raw sunflower stalks. Sun et al. (1995) have reported a protein solubilisation of about 38 %, by soaking 2.5 g of wheat straw in 100 mL of NaOH solution (1.5% NaOH) at 20°C for 6 h. Thermo-chemical pretreatments were also effective in uronic acids removal, originated from hemicelluloses and pectins, and their solubilization was complete for all pretreatments at 170°C. According to Chandel et al. (2011), hemicelluloses and pectin are binded to cellulose to form a network of cross-linked fibres. Removal of uronic acids can increase the accessibility of enzymes to hemicelluloses and cellulose (Pakarinen et al., 2012b). In contrast to oxidative and alkaline pretreatments at 55°C, a high level of hemicelluloses removal was observed for thermal pretreatment at 170°C, with or without acid reagent (Figure IV.1).

The chemical pretreatments were not effective in removing cellulose as the highest removal of 12% was observed at 170°C with HCl. Such results were also obtained by Liu et al., (2009) with corn stover. These authors found that pretreatment at 140°C for 20 min in the presence of 0.1 M $FeCl_3$, resulted in removal

of 91% of the hemicelluloses but only of 9% of the cellulose. Dilute sulfuric acid treatment has been used successfully to hydrolyze hemicelluloses to sugars with high yields (Mosier et al., 2005). Generally, acidic pretreatments are known to enhance degradation of xylans which are the main components of hemicelluloses (Nizami et al., 2010).

Oxidative and alkaline pretreatments were more efficient than acid pretreatments for lignin removal, with 30, 35, and 36% for $Ca(OH)_2$, H_2O_2 and NaOH, respectively, compared to 24 and 27% for $FeCl_3$ and HCl, respectively (Figure IV.1). Alkaline and oxidative pretreatments have been shown to be efficient in lignin removal by preserving most of the carbohydrates, in particular cellulose (Zhu et al., 2010a; Taherzadeh and Karimi, 2008). Partial delignification of 24% was obtained by further increasing the temperature to 170°C, but at this temperature, the addition of acid (HCl or $FeCl_3$) did not lead to further delignification. Guo et al. (2011) investigated different pretreatment strategies for corn stalk and concluded that acid treatment was effective in hemicelluloses removal while alkaline pretreatment led to a significant decrease in lignin content. It was also recorded that during this thermal pretreatment at high temperature with or without acid reagent, in addition to soluble sugars, furans derivatives such as furfural and 5-hydroxyl-methylfurfural (5-HMF) can be generated (Larsson et al., 1999). The furfural and 5-HMF contents were determined for conditions applied in this study (Table IV.1). Formation of furfural was observed for acidic pretreatments, whereas at 170°C, only 0.7 g / 100 g VS of furfural were recorded, which is in accordance with the results obtained by Diaz et al. (2011).

Table IV. 1 *Concentrations of furfural and 5-hydroxylmethyl-furfural in the liquid fraction of pretreated samples.*

	Liquid fraction (g / 100g initial VS)	
Conditions	**5-HMF**	**Furfural**
Raw	-	-
24 h, 55°C	0	0
24 h, 55°C, 4 % NaOH	0	0
24 h, 55°C, 4 % H_20_2	0	0
24 h, 55°C, 4 % $Ca(OH)_2$	0	0
1h, 170°C	0	0.7 (±0.02)
1h, 170°C, 10 % $FeCl_3$	0.3 (±0.02)	2.4 (±0.03)
1h, 170°C, 4 % HCl	0.4 (±0.00)	4.1 (±0.00)

Chemical composition changes during thermo-chemical pretreatments were also monitored using FT-IR spectra. The fingerprint regions of the FT-IR spectra of raw and pretreated sunflower stalks are presented in Figure IV.2.

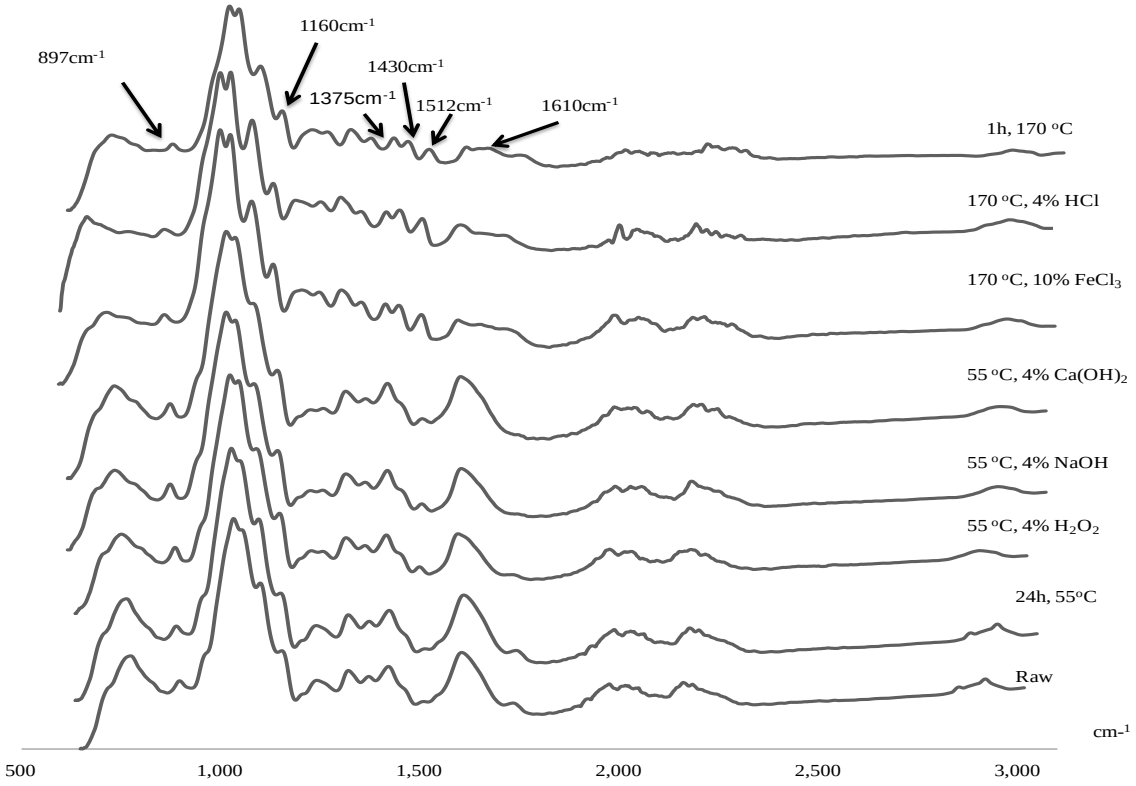

Figure IV. 2 *Fingerprint region (600-3000 cm^{-1}) of the FTIR spectra of raw and pretreated NK-Kondi sunflower stalks.*

The peaks were assigned as follows: 1610 cm^{-1} to holocelluloses (Shafiei et al., 2010); 1512 cm^{-1} is characteristic of aromatic skeletal vibration C=C of lignin; 1430 cm^{-1} assigned to C-H deformation in cellulose; 1375 cm^{-1} assigned to deformation in cellulose and hemicelluloses (Pandey and Pitman., 2003); 1160 cm^{-1} assigned to C-O-C vibration in holocelluloses and 897 cm^{-1} to CH deformation in cellulose (Yang et al., 2009). The peak at 1610 cm^{-1} assigned to cellulose and hemicelluloses decreased significantly with the increase of temperature (170°C), indicating that part of the holocelluloses was solubilized. Moreover, the intensity of the peak at 1512 cm^{-1} was higher after thermal (170°C) and acidic thermal pretreatments (170°C, HCl and 170°C, $FeCl_3$) than for raw sunflower stalks, indicating that the

relative content of lignin in sunflower stalks increased after thermal and acidic pretreatment. The previous observations on spectra can be quantified using the H lignin/H carbohydrates ratio which shows the relative intensity of lignin peaks at 1512 cm^{-1} as opposed to carbohydrates peaks at 1610, 1430, 1375, 1160 and 897 cm^{-1}. An increase in this ratio was observed after thermal-pretreatment at 170°C and for acidic conditions, which means that during such pretreatment more holocelluloses were removed than lignin (Table IV-2). The ratio of H lignin / H carbohydrates increased from 0.11 (raw sunflower stalk) to 0.16 (170 °C, 4% HCl, 1 h). Such results are in accordance with the observations on chemical composition recorded previously showing that at 170°C, 24 h, 4% HCl, all the hemicelluloses were removed.

2.2 Impact of thermo-chemical pretreatments on crystallinity of cellulose

The LOI values are presented in Table IV.2. A small decrease in the LOI ratio was noticed for acidic pretreatment (HCl and $FeCl_3$), indicating a small decrease of crystallinity of cellulose in sunflower stalks. The crystalline cellulose content was then determined using equation II-5. No significant changes in crystalline cellulose were observed after alkaline and oxidative pretreatments. With acidic pretreatments, a small decrease in crystalline cellulose was noticed as 5.3 and 14 % of crystalline cellulose removal were observed for 170°C, 1 h, 4% $FeCl_3$ and 170°C, 1 h, 4% HCl, respectively. Similar trends were noticed for the amorphous part as all thermo chemical pretreatments were not found efficient in removal of amorphous celluloses. Indeed, the highest removal of amorphous cellulose of 6% was noticed after pretreatment at 170°C, 1 h, 4% HCl.

Table IV. 2 *Lateral Order Index (LOI), crystalline cellulose (% initial VS), amorphous cellulose (% initial VS) and H lignin / H carbohydrates ratio for raw and pretreated "NK-Kondi" sunflower stalks.*

Conditions	LOI	Crystalline cellulose (% initial VS)	Amorphous cellulose (% initial VS)	H Lignin / H carbohydrates
Raw	1.22	18.7	15.3	0.11
24 h, 55°C	1.2	18.6	14.5	0.11
24 h, 55°C, 4 % NaOH	1.21	18.8	15.5	0.11
24 h, 55°C, 4 % H_20_2	1.26	19.1	15.4	0.11
24 h, 55°C, 4 % $Ca(OH)_2$	1.15	19.3	15.4	0.12
1h, 170°C	1.21	18.5	15.4	0.14
1h, 170°C, 10 % $FeCl_3$	1.16	17.7	15.2	0.15
1h, 170°C, 4 % HCl	1.1	16	14.4	0.16

3. Impact of enzymatic and combined alkaline-enzymatic pretreatments

As previously shown, alkaline pretreatment (55°C, 24 h, 4% NaOH) was found the more efficient in lignin removal and thus was selected for perform combined chemical-enzymatic pretreatments. Indeed, generally lignin removal permits to enhance the enzymatic hydrolysis (de Vrije et al., 2002). Consequently, enzymatic hydrolysis was performed on raw sunflower stalks and on the residual solid of alkaline-pretreated sunflower stalks (55°C, 24 h, 4% NaOH). Composition of sunflower stalks and solid alkaline-pretreated sunflower stalks expressed in % VS are presented in Figure IV.3. It has to be noticed that this experiment series was carried out with sunflower stalks variety "Serin 1" which was different from section IV.2. First, "Serin 1" sunflower stalks were composed of proteins, uronic acids, hemicelluloses, cellulose and lignin at, respectively, 2.5%, 2.2%, 11.4 %, 25.1 % and 32.5 % VS. Residual solid of alkaline-pretreated sunflower stalk was composed of proteins, uronic acids, hemicelluloses, cellulose and lignin at, respectively, 2.4%, 1.1%, 11.4 %, 31.3 % and 32.2 % VS.

In this part the effect of enzymatic hydrolysis on the sugars solubilisation of residual solid of alkaline-pretreated and untreated sunflower stalks was investigated. Enzymatic pretreatment on sunflower stalks led to low solubilization with respectively 11 % and 34 % of hemicelluloses and cellulose removals. Enzymatic pretreatment on residual solid of alkaline-pretreated sunflower stalks increased solubilization of holocelluloses as 45 % and 55 % of cellulose and hemicelluloses removals were respectively observed. Several factors can explain this increase of enzymatic hydrolysis after alkaline pretreatment. The first one

is the increase of accessible surface area (SA in m^2 g^{-1} TS) and volume pores (Vp in cm^3 g^{-1}TS) observed after thermo-alkaline pretreatment. A small increase of pore volume after alkaline pretreatment from 0.083 cm^3 g^{-1}TS (sunflower stalks) to 0.106 cm^3 g^{-1}TS (solid alkaline-pretreated sunflower stalks) was evaluated (Table IV.3). Accessible surface area were slightly increased from 1.55 m^2g^{-1} TS (sunflower stalks) to 1.59 m^2 g^{-1} TS (solid alkaline-pretreated sunflower stalks). However such results have to be considered with some precaution as accessible surface area and porosity experiment were performed only in one assay.

Table IV. 3 *Structural features (accessible surface area and pores volumes for raw «Serin » sunflower stalks and for the solid fraction of alkaline pretreated sunflower stalks.*

	Structural features	
	SA (m^2/g TS)	**Vp (cm^3/g TS)**
Raw « Serin » sunflower stalks	1.55	0.083
Residual solid of alkaline pretreatment (55°C, 24 h, 4% NaOH)	1.59	0.106

Gharpuray et al. (1983) observed an increase of the accessible surface area from 0.64 to 1.7 m^2 g^{-1} TS by pretreating wheat straw at 100°C with 10% NaOH (w/w) during 30 min. Gharpuray et al. (1983) have shown that specific surface area can affect the digestibility of biomass: an increase in accessible surface area resulted in higher hydrolysis yield. However the small increase of accessible surface area (+2.5%) and increase in pore volumes (+27%) between raw sunflower stalks and solid fraction of sunflower stalks pretreated at 55°C, 24 h, 4% NaOH suggests that other factors affected the enhancement of hydrolysis yield. Among them, removal of uronic acids observed after thermo alkaline pretreatment (55°C, 24 h, 4 %NaOH) could improve the enzymatic hydrolysis. Indeed, Pakirinen et al. (2012) have shown that the removal of pectin (polymer of galacturonic acids) present in hemp can increase the enzymatic hydrolysis by 26%. Moreover, physical distribution and composition of lignin can play an important role for enzyme accessibility and the digestibility of biomass and during thermo alkaline pretreatments, a physical redistribution of lignin could occur and the composition of lignin could change (Barakat et al., 2007).

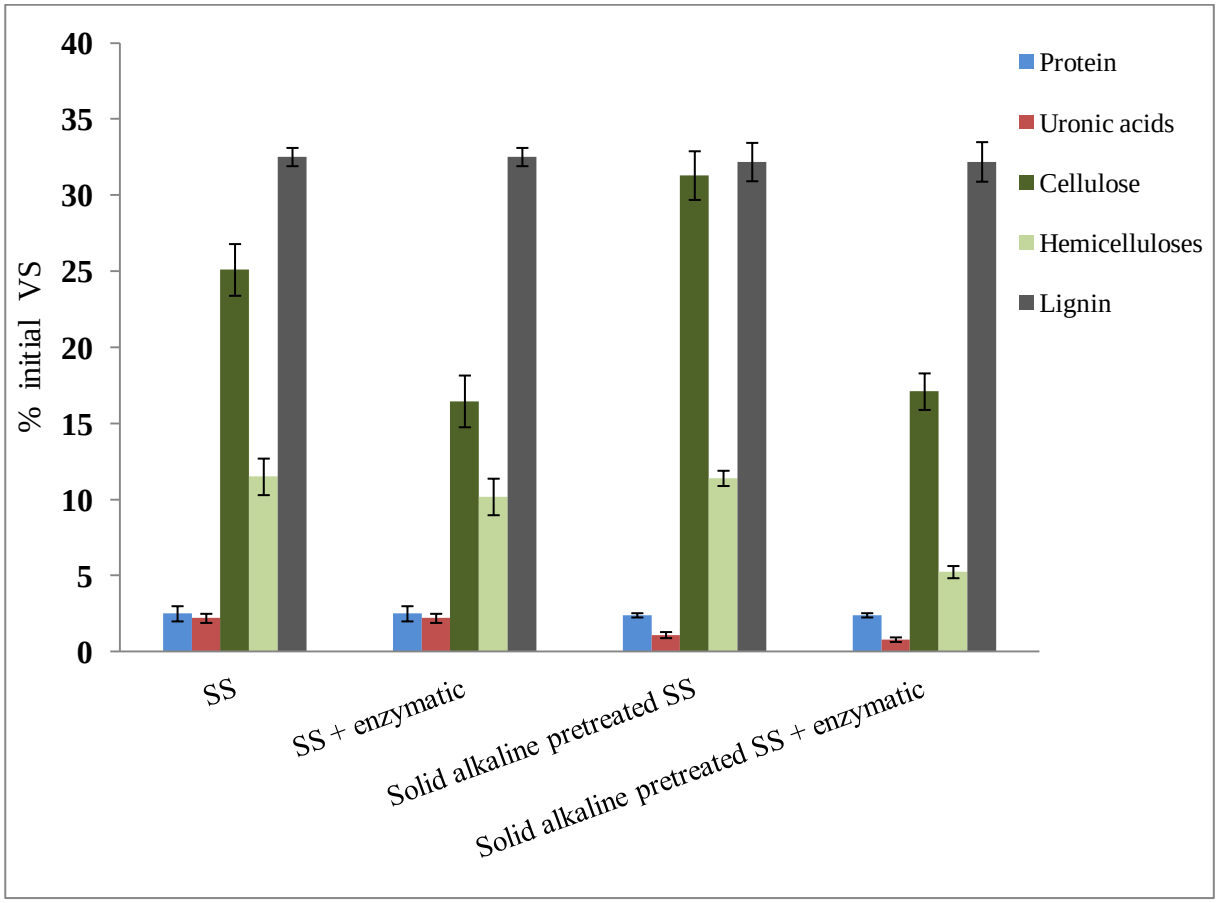

Figure IV. 3 *Compositional features of "Serin" sunflower stalks (SS), enzymatic pretreated sunflower stalk and solid alkaline pretreated sunflower stalks with or without enzymatic hydrolysis. Values correspond to means of two replicates of independent values ± standard deviation (bar errors).*

4. Conclusions

The effects of thermo-chemical pretreatments on lignocellulosic structure of sunflower stalks are summarized in figure IV.4. Acid pretreatments ($FeCl_3$ and HCl) at 170°C solubilized more than 90 % of hemicelluloses and uronic acids. Alkaline and oxidative pretreatments were more effective in dissolving lignin with 30, 35, and 36% lignin removal respectively for $Ca(OH)_2$, H_2O_2 and NaOH. However, no pretreatment was effective in reducing the crystallinity of cellulose as the best removals of crystalline cellulose of 5.3 and 14 % were observed for 170°C, 1 h, 4% $FeCl_3$ and 170°C, 1 h, 4% HCl, respectively. Combined alkaline-enzymatic pretreatment permitted to increase the enzymatic accessibility to holocelluloses and thus their further conversion into soluble sugars. Indeed, 45 % and 54 % removals respectively for cellulose and hemicelluloses were noticed by combined alkaline-enzymatic pretreatments.

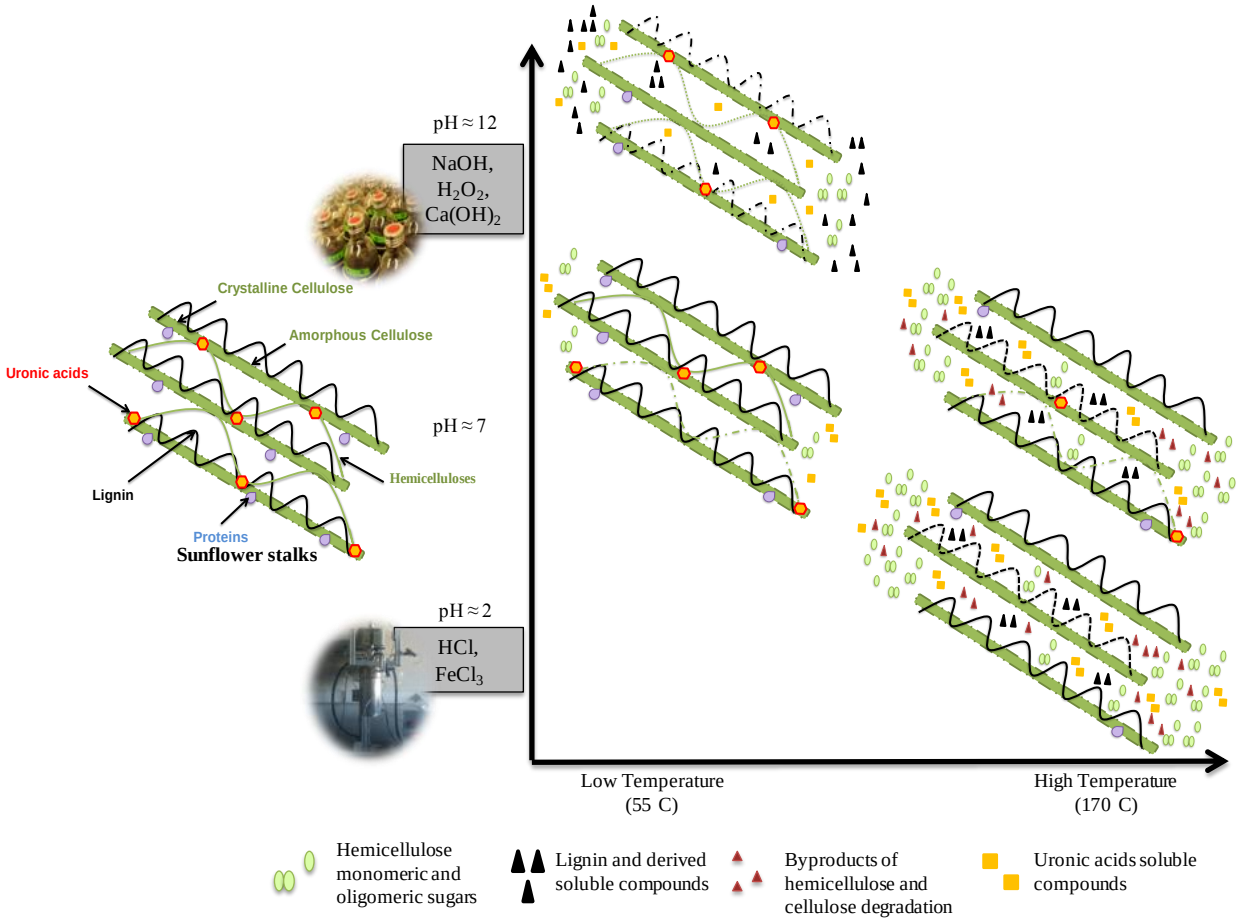

Figure IV. 4 *Overall scheme of the effects of thermo-chemical pretreatments on chemical composition of sunflower stalks adapted from Pedersen and Meyer (2010). Effect of thermo-chemical pretreatments on protein and cellulose are not represented.*

According to the results of both previous and present chapter, pretreatment strategies will be considered in chapter V and VI to enhance both hydrogen and methane production from sunflower stalks. As for hydrogen production, the increase of soluble carbohydrates content is the key parameter: acid and combined alkaline-enzymatic pretreatments which were found efficient in hydrolyzing hemicelluloses and part of cellulose will be considered for improvement of hydrogen potential.

Several factors were found to influence the methane production of lignocellulosic substrates such as reducing lignin and increasing soluble carbohydrates contents and in a lower extent reducing crystalline cellulose content. Thus, all kinds of thermo-chemical pretreatments previously investigated will be considered to enhance methane potential of sunflower stalks.

Chapter V. Thermo-chemical and enzymatic pretreatments to enhance biohydrogen production from sunflower stalks

Introduction

In this chapter, different pretreatments strategies to enhance biohydrogen production from "Serin 1" sunflower stalks were investigated. In chapter III, it was previously found that an increase in soluble sugar concentrations was a key factor to enhance hydrogen production from lignocellulosic residues. Since acidic pretreatments and combined alkaline-enzymatic pretreatments were found efficient in solubilizing holocelluloses (chapter IV), they were here considered to determine whether such pretreatment could further enhance biohydrogen production from "Serin 1" sunflower stalks.

First, the effect of the operational conditions (acid concentration, temperature) of dilute-acid pretreatments was investigated on biohydrogen production from sunflower stalks. For this, biohydrogen production was assessed in batch tests with an experimental composite design (temperature range of 142-198°C, acid concentration range of 0% – 4% HCl (w/w), time set up at 30 min). Then, to analyse the impact of the by-product concentrations (furan derivatives and phenolic compounds) on the hydrogen production microbial pathways, a gradual increase of added volumes (3.75%, 7.5%, 15% and 35% (v/v)) of dilute acid hydrolysate (170°C, 4% HCl) was tested with glucose as sole carbon source.

In the second part of this chapter, enzymatic pretreatments (a mix of cellulase, xylanase and β-glucosidase) alone or combined with alkaline pretreatments (55°C, 24 h, 4% NaOH) were investigated to enhance hydrogen production from "Serin" sunflower stalks. In the case of combined enzymatic–alkaline pretreatments, sunflower stalks were pretreated at 55°C, for 24 h, with 4% NaOH, and then the liquid fraction was separated from the solid fraction through filtration. Enzymatic pretreatment was performed only on the solid alkaline-pretreated fraction. So far, alkaline pretreatment followed by enzymatic hydrolysis has been mainly studied in a purpose of bioethanol production (Rabelo et al., 2011; Sharma et al., 2002). To our knowledge, only one study was reported on combined alkaline and enzymatic pretreatment to enhance biohydrogen production of lignocellulosic residues, by using anaerobic mixed cultures (Chairattanamanokorn et al., 2009).

1. Acidic pretreatments

2.1 Effect of dilute-acid pretreatment on hydrogen production

In the control batch test operated with untreated sunflower stalks "Serin", a hydrogen yield of 2.3 ± 0.9 mL H_2 g^{-1} VS was obtained after ten days of fermentation. Interestingly, for all the dilute-acid pretreatment conditions tested in the experimental design, no hydrogen production was observed. This result suggests that strong inhibitory compounds were released during the dilute acid pretreatments of the sunflower stalks whatever the temperature, ranging from 142-198°C, or the acid concentration, ranging from 0 to 4 g HCl per 100 g TS. These results are in agreement with Fangkum and Reungsang (2011) who observed lower hydrogen production from dilute-acid sugarcane bagasse hydrolysate (10 g L^{-1} of reducing sugars) of 1.48 mol H_2 mol^{-1} sugars compared to the control containing only 10 g L^{-1} of reducing sugars (2.49 mol H_2 mol^{-1} sugars). In relation with results obtained on methane production (chapter VI), we decided to further investigate the inhibition phenomena issued from the dilute acid hydrolysate at 170°C, 4% HCl, as no inhibition was observed for methane production under similar pretreatment conditions.

2.2 Effect of dilute-acid pretreatment on generation of undesirable byproducts

The composition of the hydrolysate obtained at 170°C, 4% HCl was determined as shown in table V.1. This pretreatment condition was highly efficient to hydrolyse hemicelluloses since about 3.14 g L^{-1} of xylose were detected in the hydrolysate, an amount equal to the degradation of 85% of the hemicelluloses initially present in the sunflower stalks. In contrast, low amounts of glucose were found in the hydrolysate, with only 0.28 g L^{-1}, an amount equal to the degradation of 3.5% of the cellulose initially present in the sunflower stalks, confirming that the dilute acid pretreatments are not efficient to hydrolyse cellulosic compounds. Besides the high amounts of xylose released in the hydrolysate, other secondary byproducts were generated: carboxylic acids such as formate (0.6 g L^{-1}) and acetate (0.8 g L^{-1}), furans derivatives such as furfural (1.15 g L^{-1}) and 5-HMF (0.13 g L^{-1}), and phenolic compounds (20.2 mg L^{-1}) (Table V.1). In general, furfural and 5-HMF are formed during pentose and hexose

thermal degradation, respectively, whereas phenolic compounds are generated from partial breakdown of lignin (Palmqvist et al., 2000). When furfural and 5-HMF are further broken down, formic acid is produced, while acetic acid results from the hydrolysis of the acetyl groups of hemicellulosic compounds (Barakat et al., 2012). Carboxylic acids such as acetate have not been reported to inhibit significantly the growth of clostridial species, which are the main hydrogen-producing bacteria found in dark fermentation. In contrast, furan derivatives and phenolic compounds can affect significantly the hydrogen-producing pathways (Cao et al., 2009; Quemeneur et al., 2012b). The release of such byproducts from dilute-acid pretreatment has been previously described in similar studies (Panagiotopoulos et al., 2011, Cao et al., 2009). The concentrations of the byproducts released in the hydrolyzate depends both on the conditions of the dilute-acid pretreatment and the origin of the lignocellulosic residues (Mussato and Roberto, 2004). Indeed, Panagiotopoulos et al (2011) showed different patterns of byproducts released after same dilute-acid pretreatment of four lignocellulosic substrates (wheat straw, barley straw, cornstalk and corncob). Scordia et al. (2012) have shown that on same lignocellulosic residue (giant reeds), the content of byproducts (ie furfural, 5-HMF and phenolic compounds) depends of the dilute-acid parameters (ie temperature, time and concentration). For instance, at the following dilute-acid pretreatment conditions (158°C, 16 min, 3,25 % oxalic acid (w/wTS)), concentrations of 0.61 g L^{-1}, 0.46 g L^{-1} and 4.6 g L^{-1} were respectively noticed for furfural, 5-HMF and phenolic compounds. By applying hard dilute-acid pretreatment conditions (190°C, 25 min, 5 % oxalic acid), an increase of the byproducts released was noticed with concentrations of 7.67 g L^{-1}, 1.48 g L^{-1} and 7.36 g L^{-1} were respectively noticed for furfural, 5-HMF and phenolic compounds.

Table V. 1 *Composition of the hydrolysate from dilute-acid pretreatment (170°C, 4% HCl) of sunflower stalks at a solid loading of 35g TS L^{-1}.*

Compounds	g L^{-1}
Glucose	0.28
Xylose	3.14
Formate	0.60
Acetate	0.81
Furfural	1.15
5-HMF	0.13
Total phenols	0.02

2.3 Effect of hydrolysate concentration (ie. byproducts) on hydrogen production

In order to investigate the impact of by-product concentrations, especially furan derivatives and phenolic compounds, on hydrogen production pathways, a gradual addition of increasing volumes of hydrolysate (3.75%, 7.5%, 15% and 35% (v/v)) was investigated on hydrogen production from glucose. The concentration of soluble sugars (glucose, xylose), metabolites (formate, acetate) and byproducts (furfural, 5-HMF, total phenols) added in BHP flasks for each condition are presented in table V.2.

Table V. 2 *Concentrations in mg L^{-1} of soluble sugars, metabolites and byproducts added in each BHP flasks according the volume added (3.75%, 7.5%, 15% and 35% (v/v)) of hydrolysate.*

Compounds	**Glc + 3.75 %**	**Glc + 7.5 %**	**Glc + 15 %**	**Glc + 35 %**
Glucose	10	21	41	96
Xylose	118	235	470	1097
Formate	22	45	89	209
Acetate	30	61	122	284
Furfural	43	86	172	402
5-HMF	5	9	19	44
Total phenols	1	2	3	7

Throughout the batch experiments, no CH_4 was detected in the gas phase, indicating that the methanogenic activity was efficiently suppressed after the heat-shock treatment of the inoculum. Biohydrogen production with glucose at 5 g L^{-1} as sole carbon source and supplemented with increasing volumes of hydrolysate (3.75%, 7.5%, 15% and 35% (v/v)) was investigated over 30 days. As glucose consumption was not total for samples supplemented with 7.5% and 35% (v/v), cumulative H_2 production curves presented in Figure V.1 were plotted in mol H_2 $mol_{glucose\_consumed}^{-1}$.

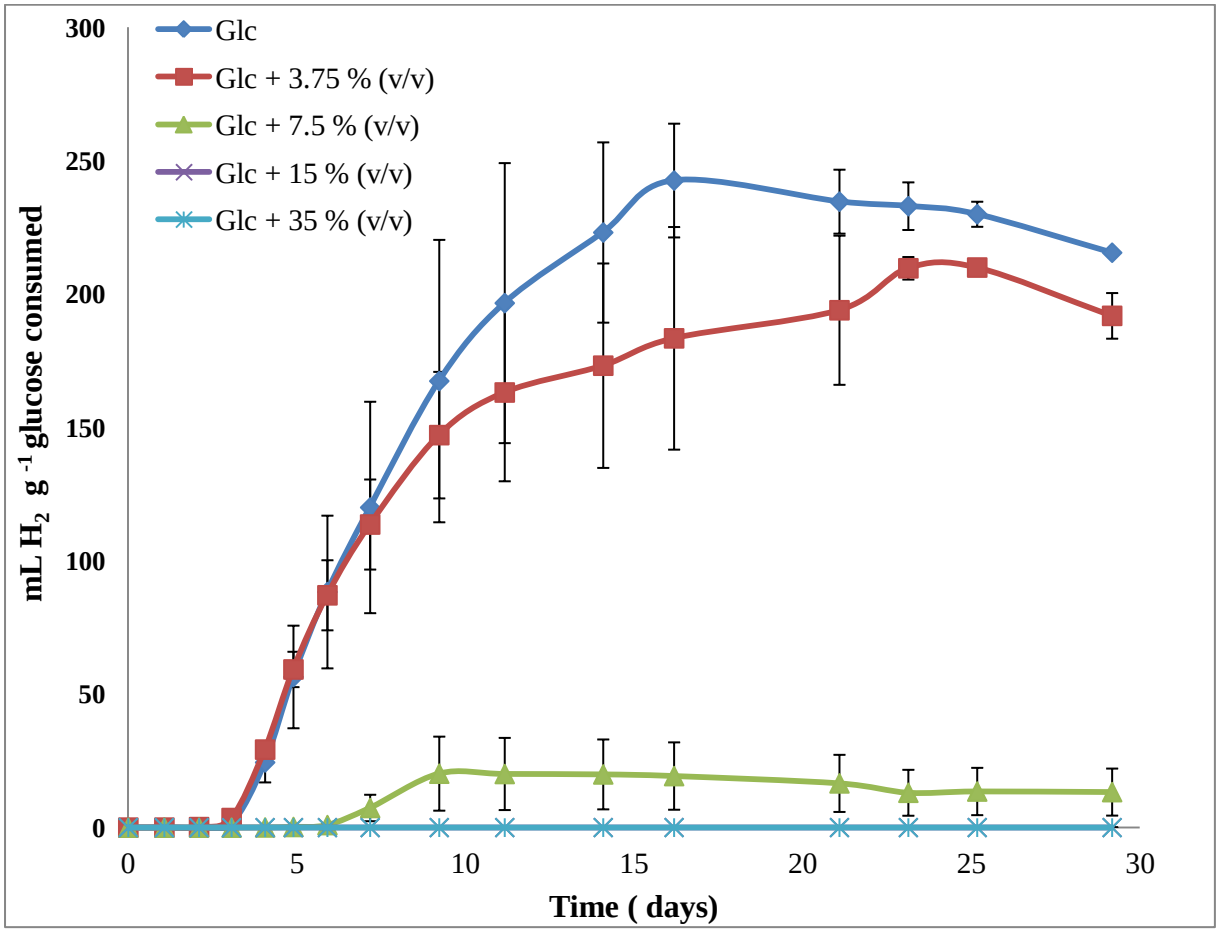

Figure V. 1 *Cumulative hydrogen curves by increasing addition volumes (3.75%, 7.5%, 15% and 35% (v/v)) of hydrolysate (170°C, 4% HCl) on glucose (5 g L^{-1}). Values correspond to means of two replicates of independent values ± standard deviation (error bars).*

To determine accurately the kinetic parameters of the H_2 production batch tests, the cumulative H_2 production (H) curves were fitted to a modified Gompertz model (Table V.3). All correlation coefficient (R^2) values were higher than 0.95, indicating that the fitted curves matched well with the experimental points. In the control, i.e. with glucose as sole carbon source and no addition of hydrolysate, H_2 production started after 3.8 days with a maximum H_2 yield of 2.04 (±0.14) mol H_2 $mol_{glucose\_consumed}^{-1}$ and a maximum hydrogen production rate of 0.32 (± 0.22) mol H_2 $mol_{glucose\ initial}^{-1}$ day^{-1}. These results are in accordance with the hydrogen yields usually reported in the literature, when using glucose as sole carbon source and mesophilic fermentative mixed cultures as inocula (Quemeneur et al., 2012b).

When 3.75 % (v/v) of hydrolysate were added to the culture medium, neither the hydrogen production yield of 1.89 (±0.08) mol H_2 $mol_{glucose\_consumed}^{-1}$, the lag phase of 2.24 (±1.47) nor the maximum H_2 production rate of 0.22 (± 0.17) $mol_{glucose\_initial}^{-1}day^{-1}$ were significantly impacted. By increasing the added volume of hydrolysate (7.5% v/v) and thus the concentration of undesirable byproducts as furfural, 5-HMF and phenolic compounds, the hydrogen fermentation performances decreased significantly to reach a H_2 yield of 0.44 (± 0.09) mol H_2 $mol_{glucose\_consumed}^{-1}$, a lag phase time of 5.82 (± 0.02) days and a H_2 production rate of 0.08 (± 0.00) $mol_{glucose\_initial}^{-1}day^{-1}$. In this assay, the corresponding byproduct concentrations were 86.2 mg L^{-1} of furfural, 9.5 mg L^{-1} of 5-HMF and 1.5 mg L^{-1} of phenolic compounds. After the addition of more than 15% (v/v) of hydrolysate, no more hydrogen production was observed after 30 days of fermentation. It was concluded that the hydrogen production pathways were highly sensitive to a slight increase of byproducts such as furans and phenolic compounds. Kongjan et al. (2009b) have noticed similar results by investigating the biohydrogen production from wheat straw hydrolysate of hydrothermal pretreatment. Indeed, they noticed a decrease of the biohydrogen potentials by increasing the volume of hydrolysate and no hydrogen production was produced at hydrolysate concentration of 30% (v/v).

Under similar conditions, Quemeneur et al. (2012b) showed that furans (furfural and 5-HMF) and phenolic compounds added separately had a negative impact on hydrogen production from xylose using mixed cultures but at a much higher concentration of 1g L^{-1}. In this study, the inhibitory concentrations observed for furans and phenolic compounds were significantly lower than the values tested by Quemeneur et al. (2012b), suggesting therefore a possible synergistic effect between furans and phenolic compounds or the presence of other unknown inhibitory compounds. This synergistic effect of byproducts (furfural, 5-HMF, phenolic compounds) have been previously observed on the bioethanol production from lignocellulosic hydrolysate (Mussatto and Roberto, 2004).

Table V. 3 *Performances of mixed-culture fermentative H_2 production in batch tests after increasing addition of hydrolysate. Values correspond to means of two replicates of independent values ± confidence intervals (error bars).*

Glucose (5gVS /L) + added hydrolysate (%(v/v))	Modified Gompertz equation parameter values				Glucose consumed (%)	Hydrogen yield (mol H_2 mol^{-1} glucose $_{consumed}$)	COD mass balance (%)	pH final
	P (mol H_2 mol^{-1} glucose $_{initial}$)	Rm (mol H_2 mol^{-1} glucose $_{initial}$ day^{-1})	λ (day)	R^2				
Glucose	2.04 (± 0.14)	0.32 (± 0.22)	3.8 (± 0.39)	0.99 (± 0.00)	100	2.04 (± 0.14)	85	3.42 (± 0.11)
Glucose + 3.75 %	1.89 (± 0.08)	0.22 (± 0.17)	2.24 (± 1.47)	0.97 (± 0.04)	100	1.89 (± 0.08)	85	3.60 (± 0.09)
Glucose + 7.5 %	0.26 (± 0.05)	0.08 (± 0.00)	5.82 (± 0.02)	0.95 (± 0.04)	63	0.44 (± 0.09)	103	3.23 (± 0.01)
Glucose + 15 %	0	0	> 30	-	100	0	102	3.67 (± 0.05)
Glucose + 35 %	0	0	> 30	-	59	0	94	4.83 (± 0.03)

2.4 Effect of hydrolysate concentration (ie. byproducts) on fermentative pathways and bacterial communities

In order to investigate the impact of these inhibitors, the metabolic patterns were determined. A mass balance in COD (Chemical Oxygen Demand) equivalents was calculated for each condition to check whether all metabolites produced were quantified (Table V.3). The COD mass balance was completed at more or less 15% to the initial COD, without considering the biomass growth, which confirms that all main microbial metabolites were quantified.

Figure V.2 shows the distribution of the soluble metabolites when the cumulative H_2 production (H) was maximal. In the control batch test operated with glucose (5 g L^{-1}) as sole carbon source and no hydrolysate, high levels of butyrate and acetate were observed, with 0.69 (±0.02) $mol_{butyrate}$ $mol_{glucose\_consumed}^{-1}$ and 0.55 (±0.01) $mol_{acetate}$ $mol_{glucose\_consumed}^{-1}$, suggesting that hydrogen was mainly produced from the acetate-butyrate fermentation pathways. In the culture supplemented with 3.75% (v/v) of hydrolysate, the results were rather similar, with butyrate, acetate and ethanol as main metabolites, at 0.60 (±0.06) $mol_{butyrate}$ $mol_{glucose\_consumed}^{-1}$, 0.5 (±0.04) $mol_{acetate}$ $mol_{glucose\_consumed}^{-1}$ and 0.37 (±0.09) $mol_{ethanol}$ $mol_{glucose\_consumed}^{-1}$, respectively. In the culture supplemented with 7.5 % (v/v) of hydrolysate, the decrease of 83% of the hydrogen yield was consistent with the low contents of butyrate (0.11 mol $mol_{glucose\_consumed}^{-1}$) and acetate (0.16 mol.mol $_{glucose\_consumed}^{-1}$), and the higher concentrations in ethanol (0.76 mol $mol_{glucose\_consumed}^{-1}$) and lactate (0.87 mol $mol_{glucose\_consumed}^{-1}$). For volumes of hydrolysate higher than 15% (v/v), only ethanol and lactate were produced. In the culture supplemented with 15 % (v/v) of hydrolysate, 0.40 (±0.00) $mol_{lactate}$ mol $_{glucose\_consumed}^{-1}$ and 1.61 (±0.02) $mol_{ethanol}$ mol $_{glucose\_consumed}^{-1}$ were generated and with 35 % (v/v) of hydrolysate, 0.08(±0.00) $mol_{lactate}$ mol $_{glucose\_consumed}^{-1}$ and 1.9 (±0.05) $mol_{ethanol}$ mol $_{glucose\_consumed}^{-1}$ were produced. Interestingly, the decrease of H_2 production occurring for added volumes of hydrolysate higher than 7.5 % (v/v) was concomitant with the accumulation of lactate and ethanol. Ethanol and lactate are known as metabolites generated in zero-hydrogen balance microbial pathways which is consistent with the absence of hydrogen production in these cases.

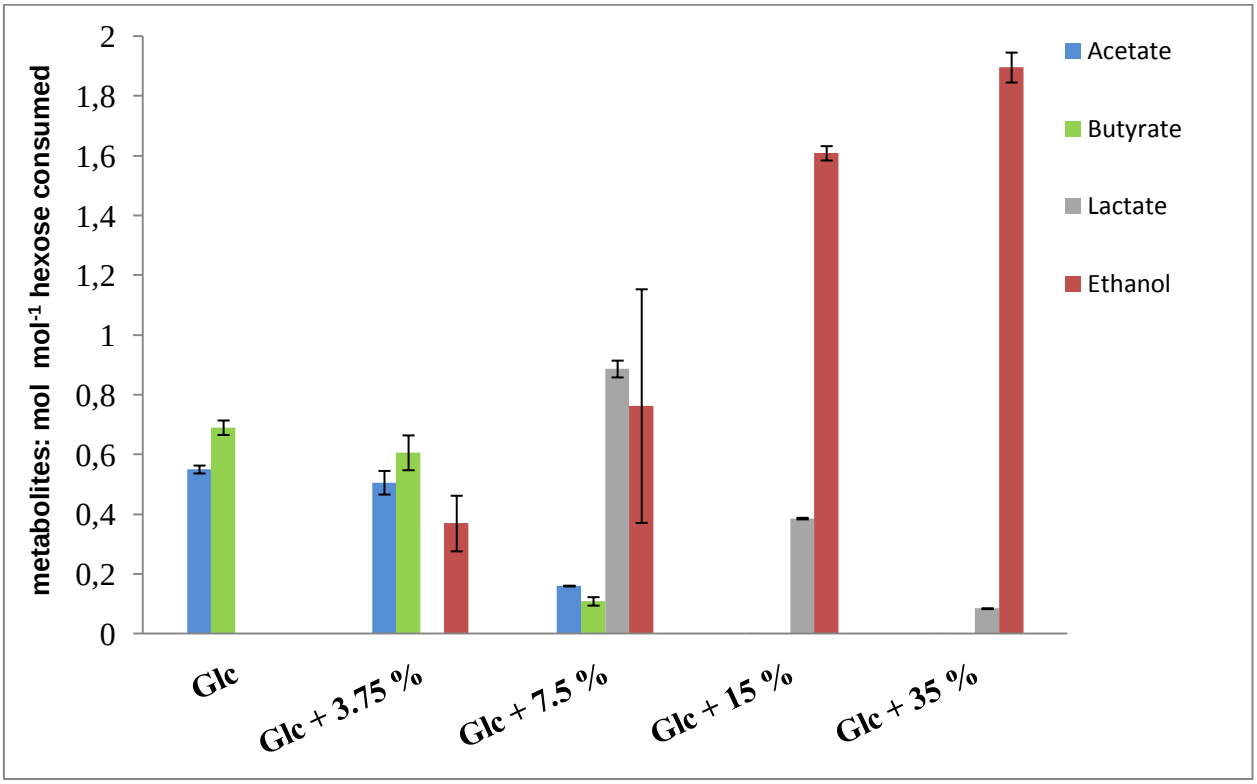

Figure V. 2 *Metabolite patterns after addition of increasing volumes of hydrolysate (3.75%, 7.5%, 15% and 35% (v/v)) in fermentative mixed cultures. Values correspond to means of two replicates of independent values ± confidence intervals (error bars) determined at Hmax time.*

To investigate the reasons why this metabolites shift from acetate/butyrate to lactate/ethanol, bacterial communities from H_2-producing mixed cultures subject to the addition of increasing volumes (3.75%, 7.5%, 15% and 35% (v/v)) of dilute acid hydrolysate (170°C, 4% HCl) were characterized using CE-SSCP fingerprinting profiles (Figure V.3). Considering the different CE-SSCP fingerprinting profiles, seven main peaks were identified and corresponded to individual bacterial species. According to Quemeneur et al., (2012b) who worked on the same anaerobic digested sludge pretreated by heat shock, bacterial species at the left of the CE-SSCP fingerprinting profiles corresponded to clostridial species whereas species at the right corresponded to non clostridial species (mainly lactic acid bacteria). Similar trend was confirmed by Guo et al. (2012) who suggested that *clostridium* species are commonly found in the left and non *clostridium* species at the right of the CE-SSCP fingerprinting profiles. Non clostridial species such as *Lactobacillus*, *Enterobacter*, etc correspond to H_2 consumers or competitors to sugars (lactic acid bacteria). Thus, according to the fingerprinting profiles, peaks 1, 2, 3 and 4 can be associated to clostridial species whereas peaks 5, 6 and 7 to

non clostridial species. The addition of increasing volumes of hydrolysate had a significant effect on the bacterial community structures as shown in Figure V.3. The profiles obtained by CE-SSCP consist of a succession of peaks where the peak area is proportional to the abundance of individual bacterial species. Thus, the relative abundance of clostridial-like species and non clostridial species was calculated considering the area of the peak from the left and from the right parts separated by the red line in the Figure V.3 of the CE-SSCP profiles, respectively.

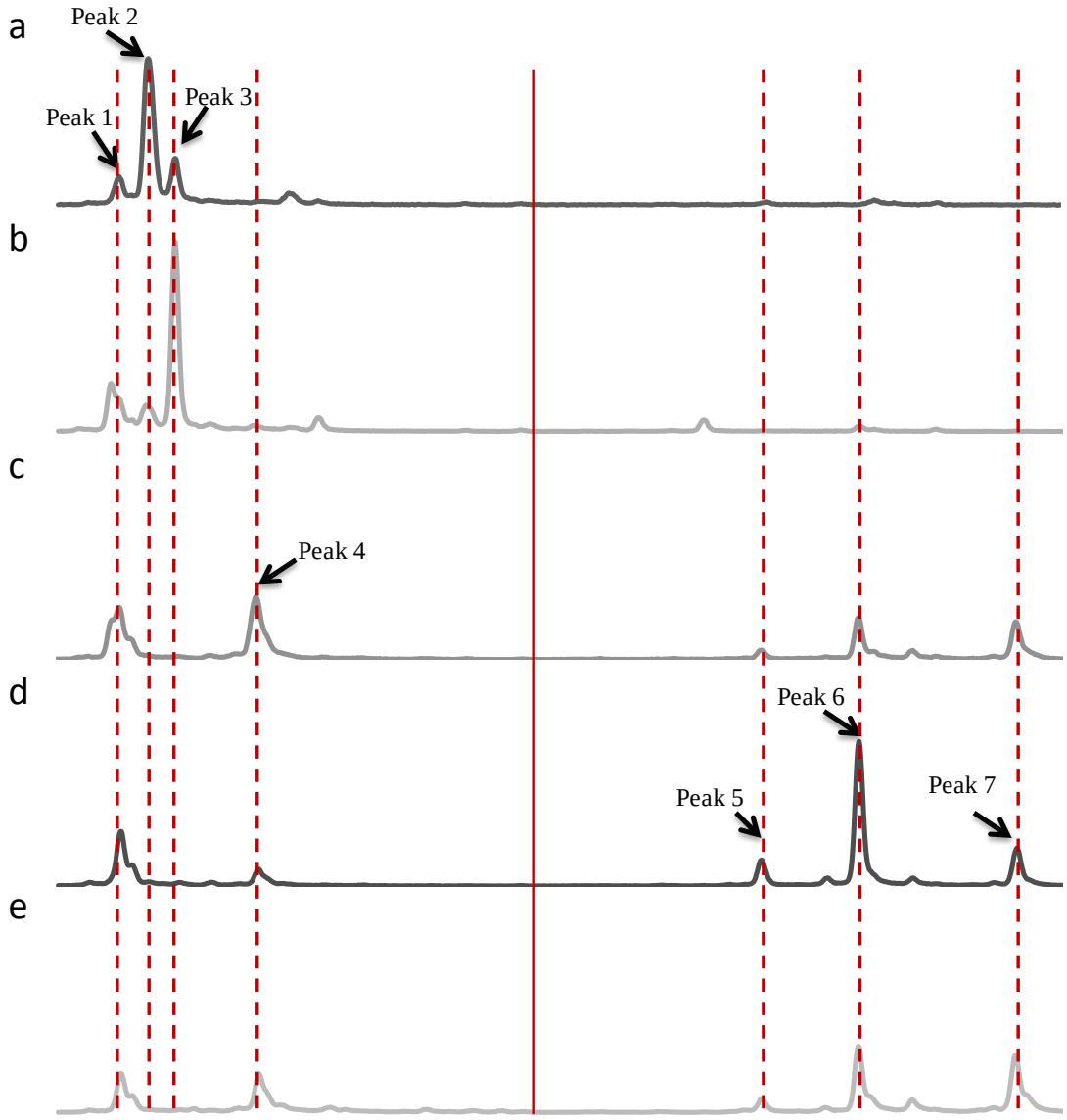

Figure V. 3 *CE-SSCP profiles based on 165 rRNA gene fragments retrieved from H_2-producing mixed cultures at gradual increase of added volumes of dilute acid hydrolysate (170°C, 4% HCl): 0% (v/v) (a), 3.75% (v/v) (b), 7.5% (v/v) (c), 15 % (v/v) (d) and 35% (v/v) (e). Each CE-SSCP profile was first aligned using an internal standard, and was then normalized. The X and Y axes of each CE-SSCP profie represent the relative peak electrophoresis migration distance and the relative peak intentsity, respectively.*

For the control, operated with glucose as sole carbon sources and no hydrolysate addition, clostridial species represented 90 % of the bacterial community with regards to only 10% of non clostridial species. Similar trend was observed after addition of 3.75 % (v/v) of hydrolysate since clostridial species represented about 90 % of the total bacterial community. For volumes of hydrolysate higher than 7.5 % (v/v), new emerging microorganisms (peak 5, 6 and 7) corresponding to non clostridial species were found. Nevertheless, with a volume of hydrolysate of 7.5 % (v/v), clostridial species were still in major abundance (61%) but reduced. This emergence of non clostridial species (39 % in relative abundance) which are H_2 consumers or competitors supported the accumulation of lactate and ethanol metabolites and the decrease in hydrogen yields previously observed. At 15 % and 35 % (v/v) of hydrolysate added, non clostridial species were in major abundance with 66 % and 57 %, respectively. Although a low but significant presence of clostridial species, no hydrogen production was observed at 15 % and 35 % (v/v) of hydrolysate added suggesting that besides the development of H_2 consumers or competitors, conditions were also stressful for the clostridial species. Fangkum and Reungsang, (2011) reported also the production of lactate and ethanol metabolites from dark fermentation of acid-hydrolysed sugarcane bagasse containing byproducts. The production of such metabolites was explained by the presence of *Clostridium ragsdalei* which is an hydrogen consumer able to produce ethanol and the production of lactate by microorganisms of the class *Bacilli viz. Lactococus lactis subsp., Lactobacillus delbrueckii, Strepto coccus pyogenes and Sporolactobacillus sp.*. To conclude, the hydrogen inhibition and the associated metabolic shift observed in this study by the addition of hydrolysate was most likely due to (i) the development of H_2 consumers or metabolic competitors such as lactic acid bacteria, and (ii) stressful conditions for hydrogen-producing bacteria.

Even though no H_2 was produced after addition of high amounts of hydrolysate, microbial activity was still observed since glucose was consumed and microbial metabolites accumulated. As dark fermentation is an intermediate stage of the anaerobic digestion process, these metabolites can be further used to produce methane whatever their accumulation patterns. This is in agreement with Barakat et al., (2012) who observed no inhibition of methane production from xylose in presence of similar byproducts (furans

derivatives and phenolics compounds) added separately at a concentration of 1 g L^{-1}. This suggests that alternative pathways to acetate or butyrate could be utilized by anaerobic bacteria to convert lignocellulosic residues in anaerobic digestion, in presence of such specific hydrogen-producing pathways inhibitors.

Nevertheless, in the concept of bio-refinery, supplementation of dark fermentation cultures by byproducts like furans and phenolic compounds can be used as strategy to turn toward other values added products such as ethanol and lactate.

2. Combined alkaline-enzymatic pretreatments

2.1 Effect of enzymatic and combined alkaline-enzymatic pretreatments on hydrogen production

In this part, the impact of enzymatic and combined alkaline-enzymatic pretreatment on the biohydrogen production of sunflower stalks was investigated. Before alkaline, enzymatic and combined alkaline-enzymatic pretreatments, "Serin" sunflower stalks were sterilized (120°C, 20min). Indeed, in the absence of sterilization, Quemeneur et al. (2012a) showed a consumption of free sugars by indigenous wheat straw microorganisms during enzymatic hydrolysis.

During dark fermentation, only H_2 and CO_2 were present in the biogas, without any detectable CH_4 suggesting that heat shock was efficient to remove methanogens in the anaerobic mixed culture used in this study. The cumulative H_2 production (H) was fitted to the modified Gompertz (Equation II.8). According to the data in Table V.4, all R^2 values were higher than 0.94, indicating that the fitted curves matched well with the experimental points.

No significant increase in hydrogen potentials (4.9 ± 2.9 mL H_2 g $_{initial}VS^{-1}$) was observed after thermo-alkaline pretreatments alone (55°C, 24 h, 4% NaOH) compared to raw sunflower stalks (2.2 ± 1 mL H_2 g^{-1} VS) as shown in Figure V.5. Cui et al. (2011) observed contradictory results by pretreating grass at 0.5% NaOH (w/v) and boiling for 30 min. In this case, a maximum cumulative hydrogen yield of 19.25 mL H_2 g^{-1} dry grass compared to the 4.39 mL H_2 g^{-1}dry grass of the untreated sample was found. Shi et al. (2010) also showed an increase in hydrogen potentials after alkaline pretreatment (0.4 % NaOH, 24 h, room temperature) of sweet sorghum stalks (127 mL H_2 g^{-1} VS vs 52 mL H_2 g^{-1} VS). Shi et al. (2010) suggested that the

enhancement of hydrogen yields nearly coincided with an increase in water soluble sugars available from alkali pretreated and raw sweet sorghum stalks, which were 2.23 and 0.86 g L^{-1}, respectively (Shi et al., 2010). Although a low but significant solubilisation of hemicellulose (23,2%) and celluloses (3,4%) occured during alkaline pretreatment as shown in chapter VI-4, a very low amount of solubilized sugars were in their monomeric form (20 mg g^{-1} VS). Recently, Quemeneur et al. (2011) showed that the structure of di- and tri-saccharides affects significantly the hydrogen production and longer chains of oligomers lead to lower hydrogen yields. Thus, the low solubilisation of carbohydrates in majority of the form of oligomers can explain the low hydrogen enhancement observed after alkaline pretreatment of sunflower stalks compared to raw sunflower stalk.

For enzymatic pretreated sunflower stalks, a cumulative hydrogen yield of 28.6 mL H_2 g^{-1} VS (± 3.6), a maximum H_2 production rate of 11.3 (± 13.7) mL H_2 g^{-1}VS.$hour^{-1}$ and a lag phase of 20.7 (± 0.7) hours were observed. Coupling thermo alkaline with enzymatic hydrolysis did not reduce the lag phase i.e. 24(±4) hours. Nonetheless, coupling thermo alkaline with enzymatic hydrolysis improved significantly the cumulative hydrogen yield to a value of 55.0 (± 3.2) mL H_2 g^{-1} VS after alkaline pretreatment at 55°C, 24 h, 4% NaOH combined with enzymatic hydrolysis.

Table V. 4 *Performances of biohydrogen production on raw, alkaline pretreated, enzymatic pretreated and combined enzymatic-alkaline pretreated "Serin" sunflower stalks.*

	Modified Gompertz equation parameter values			
Conditions	**P (mL H_2 g^{-1}VS)**	**Rm (mL H_2 g^{-1} VS $hour^{-1}$)**	**λ (hours)**	**R^2**
Raw	2.2 (± 1)	1.9 (± 0.8)	29 (± 15)	0.94 (± 0.06)
55°C, 24 h, 4 % NaOH	4.9 (± 2.9)	2.7 (± 1.1)	26 (± 10)	0.99 (± 0.00)
Raw + Enzymes	28.6 (± 3.6)	11.3 (± 13.7)	20.7 (± 0.7)	0.99 (± 0.00)
55°C, 24 h, 4 % NaOH + Enzymes	55.0 (± 3.2)	20.1 (± 22.6)	24 (± 4)	0.97 (± 0.00)

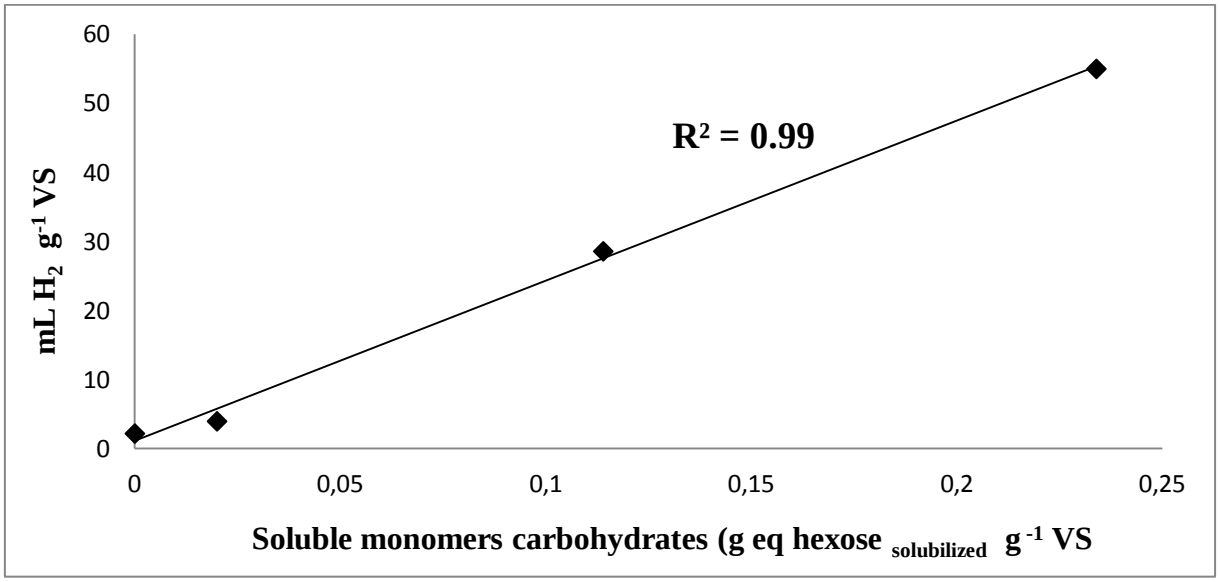

Figure V. 4 *Correlation between hydrogen production (mL H_2 g^{-1} $_{initial}$ VS) and soluble monomers carbohydrates (g eq hexose $_{solubilized}$ g^{-1} VS).*

Moreover, a high correlation (R^2 = 0.99) was found between the hydrogen yield and the content in soluble monomeric carbohydrates expressed in g eq hexose $_{solubilised}$ g^{-1} VS (Figure V.4) This result is in accordance with previous observations reported in chapter III that suggest that hydrogen yields from lignocellulosic materials are highly dependent of the soluble carbohydrates content.

In the case of combined alkaline-enzymatic pretreated sunflower stalks, the liquid and solid fractions were separated at the end of the alkaline pretreatment and the enzymatic pretreatment step was carried out only on the solid fraction. To assess the advantage of combining a thermo-alkaline pretreatment with an enzymatic hydrolysis, H_2 production from the liquid fraction and enzymatic pretreated solid fraction were both evaluated (Figure V.5). Results are expressed in mL H_2 g^{-1} $VS_{initial}$ (ie. VS before alkaline pretreatment) whereas in Table V.4 and figure V.4, results are reported to VS actually introduced in BHP test (ie solid VS after alkaline pretreatment)

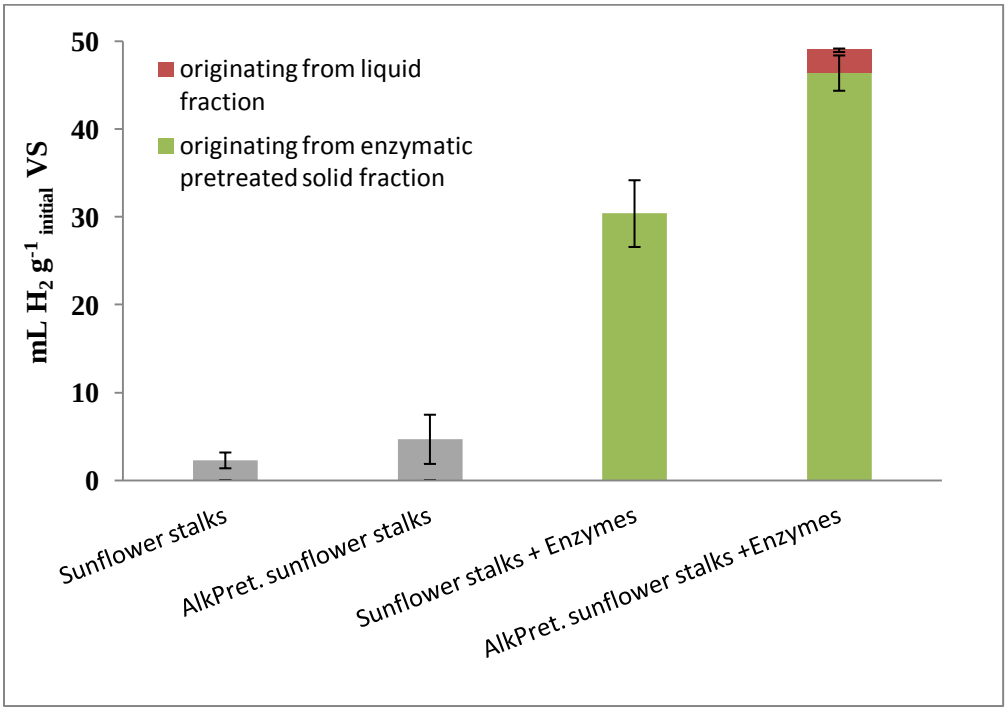

Figure V. 5 *Hydrogen production (mL H_2 g^{-1} $_{initial}$ VS) of raw sunflower stalks with or without enzymatic hydrolysis, alkaline pretreated (AlkPre.) sunflowers stalks and solid alkaline pretreated residue with enzymatic hydrolysis.*

A hydrogen potential of 30.4 mL H_2 g^{-1} $_{initial}$ VS was observed after enzymatic pretreatment, which was 13-fold greater than with raw sunflower stalks (2.3 ± 0.9 mL H_2 g^{-1} $_{initial}$ VS). These results are consistent with those of Quemeneur et al. (2012a) who showed an substantial increase in hydrogen yields after enzymatic hydrolysis (5 mg proteins g^{-1} wheat straw) of sterilized wheat straw (18 ± 2 mL H_2 g^{-1} VS vs 8.8 ± 0.8 mL H_2 g^{-1} VS).

When considering thermo-alkaline pretreatment combined with enzymatic hydrolysis, the hydrogen yield was 21-fold higher than the untreated sunflower stalks. Coupling thermo-alkaline and enzymatic treatments led to a significant increase in hydrogen production from 30.4 mL H_2 g^{-1} $_{initial}$ VS (enzymatic pretreatment alone) to 49 mL H_2 g^{-1} $_{initial}$ VS. Alkaline pretreatment prior to enzymatic hydrolysis permitted to increase the enzymatic accessibility to holocelluloses for further solubilization into monomeric sugars. Previously, Chairattanamanokorn et al. (2009) used combined alkaline pretreatment (4% NaOH (w/v) at 100°C for 2 h) and enzymatic hydrolysis (commercial cellulase from *Trichoderma Reesei* at 20 U g^{-1} substrate). A hydrogen yield of 300 mL H_2 g^{-1} VS was observed compared to 31 mL H_2 g^{-1} VS with enzymatic hydrolysis alone

(Chairattanamanokorn et al., 2009). However, to make the process economically viable, the outlets of H_2 fermentation rich in metabolites such as acetate and butyrate should also be considered for further utilization in photo-fermentation process producing hydrogen, or for methane production by anaerobic digestion (Hawkes et al., 2007; Nath et al., 2004).

2.2 Effect of enzymatic and combined alkaline-enzymatic pretreatments on fermentative pathways and bacterial communities.

The metabolic patterns observed during dark fermentation of raw, alkaline pretreated, enzymatically and combined alkaline-enzymatic pretreated sunflower stalks are presented in Figure V.6. Among the metabolites found, only acetate and butyrate were produced suggesting that H_2 was produced exclusively through the acetate-butyrate fermentation pathways. Hydrogen-consuming pathways, ie. propionate, or zero-hydrogen balance pathways, ie. lactate and ethanol, did not occur. When the maximum cumulative hydrogen amount was reached, 0.14 g $L^{-1}$of acetate and 0.10 g L^{-1} of butyrate were produced from the raw sunflower stalks, 0.78 g $L^{-1}$of acetate and 0.22 g $L^{-1}$of butyrate from the alkaline pretreated sunflower stalks, 0.64 g $L^{-1}$of acetate and 0.64 g $L^{-1}$of butyrate from the enzymatically pretreated sunflower stalks and 1.15 g $L^{-1}$of acetate and 1.26 g $L^{-1}$of butyrate from the combined alkaline-enzymatic pretreated sunflower stalks. The high amount of acetate after dark fermentation of alkaline pretreated sunflower stalks compared to the low hydrogen yield obtained can be explained by solubilization of acetyl groups of the lignocellulosic matrix into acetate during alkaline pretreatment. Thus, an amount of acetate coming from alkaline pretreatment was certaintly introduced in the hydrogen batch. The production of butyrate as well as acetate as end-products is typical of dark fermentation from carbohydrates rich-substrates (Guo et al., 2010). Similarly, Quemeneur et al. (2012a) reported that acetate and butyrate were the dominant metabolites produced during mesophilic H_2 production from wheat straw using as inoculum heat pretreated (90°C, 10 min) mesophilic anaerobically-digested sludge.

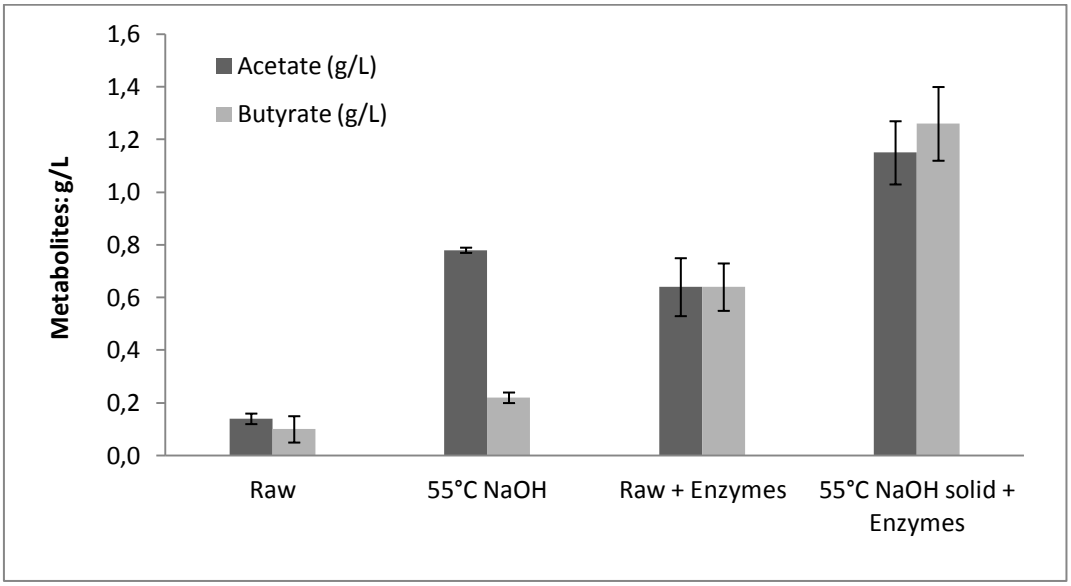

Figure V. 6 *Metabolites formation during dark H_2 mixed culture fermentation of raw, alkaline pretreated, enzymatically pretreated and enzymatic pretreatment on solid residue of alkaline pretreated sunflower stalks.*

During dark H_2 fermentation of enzymatically and combined enzymatic-alkaline pretreated sunflower stalks, it was shown that glucose was first consumed and, once glucose was completely exhausted, xylose was utilized by the microorganisms (Figure V.7). It seemed that a diauxic effect occurred during dark H_2 fermentation using anaerobic mixed culture. Such effects were previously reported on ethanol production from glucose-xylose mixture using pure cultures, ie. *Saccharomyces cerevisiae* and *E.coli* (Kuyper et al., 2005; Hanly and Henson, 2010). When grown on a mixture of sugars such as those obtained from plant biomass, wild type of *E.coli* exhibits sequential consumption of them, which is manifested as diauxic growth (Dellomonaco et al., 2010). Diauxic effect commonly occurs when cells are exposed to multiple carbon sources and bacteria display a catabolic repressive effect that does not permit the simultaneous consumption of all sugars, i.e. sugars are sequentially consumed resulting in two successive exponential growth phases that are separated by intermediate lag phases (Monod, 1942). According to Riyanti and Rogers (2009), lag phase corresponds to the time that microbial cells produce the enzymes needed to metabolize the second type of sugars. This diauxic effect results from carbon catabolite repression which is a regulatory mechanism of gene expression and the metabolism of a secondary substrate is prevented by the presence of preferred

substrates (Stulke and Hillen, 1999). According to Dellomonaco et al. (2010), glucose is the preferred substrate for many microorganisms ie. *E.coli*, and is thus responsible for catabolic repression which is commonly called the "glucose effect".

To support the hypothesis of a diauxic effect, a bacterial analysis from H_2-producing mixed cultures was performed at two experimental times: 40 h and 92 h, corresponding to the end of glucose and xylose consumption, respectively. Representative bacteria CE-SSCP fingerprinting profiles based on 165 rDNA gene fragments retrieved from H_2-producing mixed cultures are presented in Figure V.7. The microbial diversity was estimated from the number of visible peaks within the CE-SSCP fingerprint profiles. For enzymatically pretreated and combined alkaline-enzymatic pretreatments from sunflower stalks, two identical peaks were present at 40 h and 92 h suggesting that same H_2-producing bacteria were present during both glucose and xylose consumption. The production of butyrate and acetate as fermentation end-products suggested that the H_2-producing bacteria were probably related to clostridial species (Quemeneur et al., 2012b). Thus a diauxic effect occurred during the consumption of glucose-xylose mixture released during enzymatically pretreated and combined alkaline-enzymatic pretreatments. Up to date, to our knowledge, it's the first time that diauxic effect was observed on biohydrogen production using sugars mixtures. In general, diauxic effect can be avoided by the use of mixed cultures instead of pure cultures as they are able to convert concomitantly a large range of mixture substrates due to their high microbial diversity and the possibility of specific individual ecological niches (Hanly and Henson, 2010). In our case, the heat shock pretreatment of the anaerobic mixed culture sludge performed to inhibit the archaeal methanogens also led to a strong selection of bacteria as only two main strains of bacteria were observed in the CE-SSCP fingerprint profiles (red arrow in Figure V.7). This observation is in accordance with Baghchehsaraee et al. (2008) that showed that heat pretreatment (80 °C or 95 °C) of inocula led to a decrease of the species diversity. Comparatively, Chaganti et al. (2012) reported much higher numbers of bacteria ranging from 114 to 164 on three granular and non-granular mesophilic cultures which were not heat pretreated. Thus, the diauxic effect observed previously can be explained by the strong selection of bacteria that occurred during heat shock pretreatment of anaerobic sludge

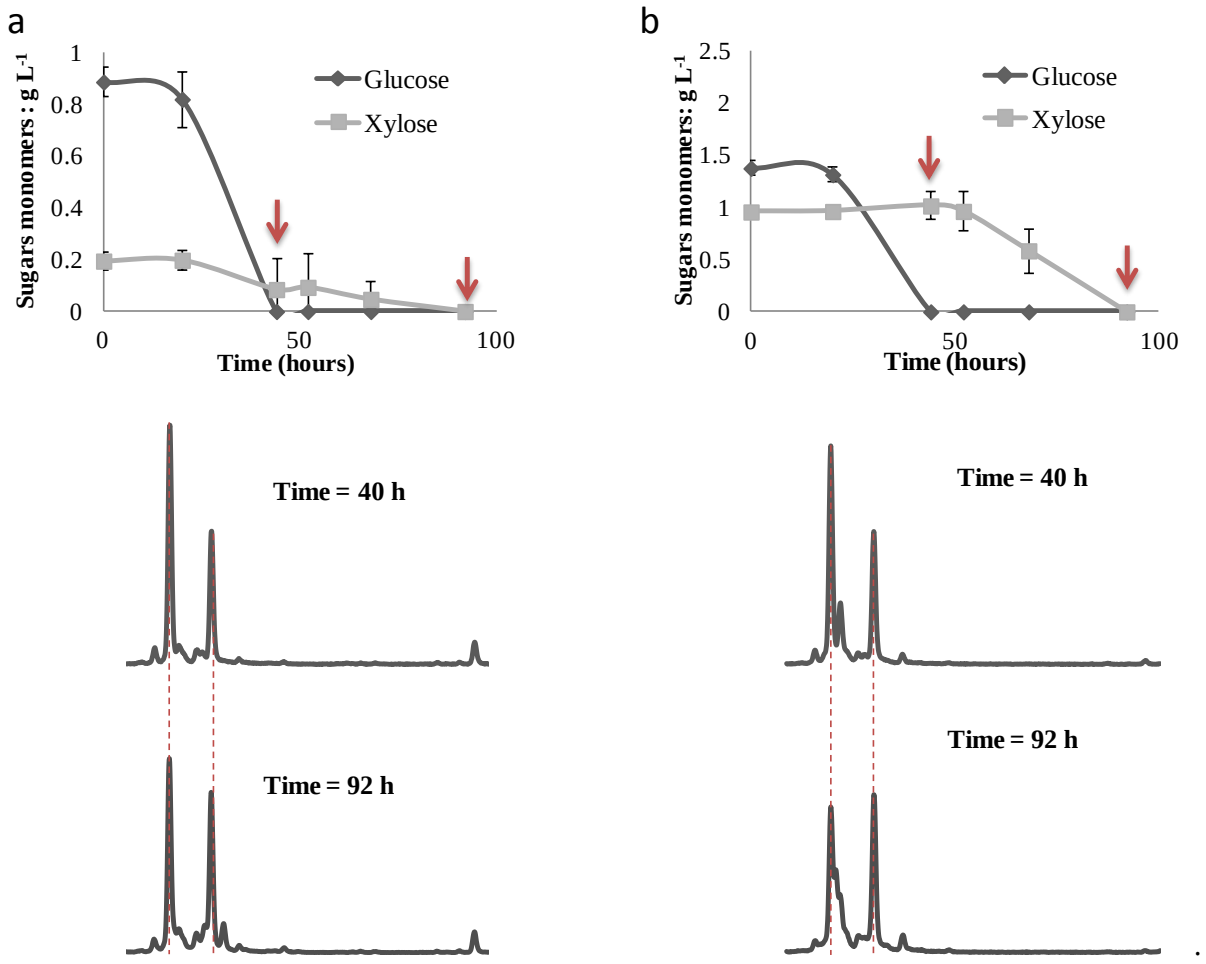

Figure V. 7 *Evolution of sugars monomers (glucose and xylose) concentration during H_2 fermentation and CE-SSCP profiles based on 165 rRNA gene fragments retrieved from H_2-producing mixed cultures at 40 h and 92 h (a) from enzymatically pretreated sunflower stalks (b) from combined alkaline-enzymatic pretreatments from sunflower stalks.*

3. Conclusions

As shown in this chapter, after dilute acid pretreatment of lignocellulosic residues (170°C, 4% HCl), byproducts such as furan derivatives (furfural (1.15 g L^{-1}) and 5-HMF (0.13 g L^{-1}) and phenolic compounds (20.2 mg L^{-1}) were generated concomitantly to the expected release of soluble sugars such as glucose (0.28 g L^{-1}) and xylose (3.14 g L^{-1}). A strong and significant inhibition of biohydrogen fermentation by these byproducts was observed. For a low concentration of hydrolysate added (7.5 % (v/v)) equivalent to only 86.2 mg L^{-1} of furfural, 9.5 mg L^{-1} of 5-HMF and 1.5 mg L^{-1} of phenolic compounds, a substantial decrease

of hydrogen production was observed by metabolic and microbial population shifts, suggesting a specific effect of the inhibitors on H_2-producing bacteria. Ethanol and lactate that are involved in zero-hydrogen balance pathways mainly accumulated, and likely resulted from the development of H_2 consumers or competitors such as lactic acid bacteria but also from stressful conditions for hydrogen-producing bacteria. In the second part, enzymatic and combined alkaline-enzymatic pretreatments significantly enhanced the hydrogen potentials with, respectively, 30.4 and 49 mL $H_2\,g^{-1}\,VS_{initial}$ compared to raw sunflower stalks (2.3 mL $H_2\,g^{-1}\,VS_{initial}$). During the consumption of xylose-glucose mixture released in the hydrolysate, a diauxic effect was observed as glucose was first consumed and only once all glucose was exhausted, xylose consumption started. However, enzymatic pretreatments present several bottlenecks for future development at industrial scale as their high cost and aseptic conditions requirement. Furthermore, to be economically viable, such process should be coupled with another process that convert metabolites mainly acetate and butyrate produced during dark H_2 fermentation. A promising way is to convert these metabolites into methane in a two-stage H_2/CH_4 process (Kongjan et al., 2010; Hawkes et al., 2007).

NB: Samples from CE-SSCP have been sent for pyrosequencing to determine the name of the bacterial species identified previously. The results are under acquisition at the time of writing.

Chapter VI. Thermo-chemical pretreatments to enhance methane production from sunflower stalks

Adapted from "Monlau et al., 2012, Comparison of seven types of thermo-chemical pretreatment on the structural features and anaerobic digestion of sunflower stalks. Bioresource Technology, 2012, 120, 241-247".

1. Introduction

In this chapter, different thermo-chemical pretreatments were investigated in order to enhance methane production from "NK-Kondi" sunflower stalks. In chapter III, it was highlighted that a decrease in lignin content, an increase in soluble carbohydrates and, in a lesser extent, a decrease in crystalline cellulose were the most important factors to enhance methane production from lignocellulosic substrates.

Due to the efficiency of acidic pretreatments in increasing solubilisation of hemicelluloses into sugar monomers and of alkaline and oxidative pretreatments in delignification (chapter IV), both pretreatments were considered to enhance methane production.

In the first part, two thermal (55°C, 170°C) and five thermo-chemical pretreatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) were carried out on "NK-Kondi" sunflower stalks and their impact on methane potential were analysed. In the second part, the effects of alkaline pretreatments conditions (time, temperature and concentration) on methane production from sunflower stalks were assessed. Best pretreatment conditions were further compared between four types of sunflower stalks.

2. Thermo-chemical pretreatments

2.1 Impact on methane potentials and methane production rate

The methane potentials of raw, thermal pretreated (55°C, 170°C) and thermo-chemical pretreated sunflower stalks (55°C, 24 h, 4% NaOH, 55°C, 24 h, 4% H_2O_2, 55°C, 24 h, 4% $Ca(OH)_2$, 170°C, 1h, 4% HCl and 170°C, 1h, 10% $FeCl_3$) were determined (Figure VI.1).

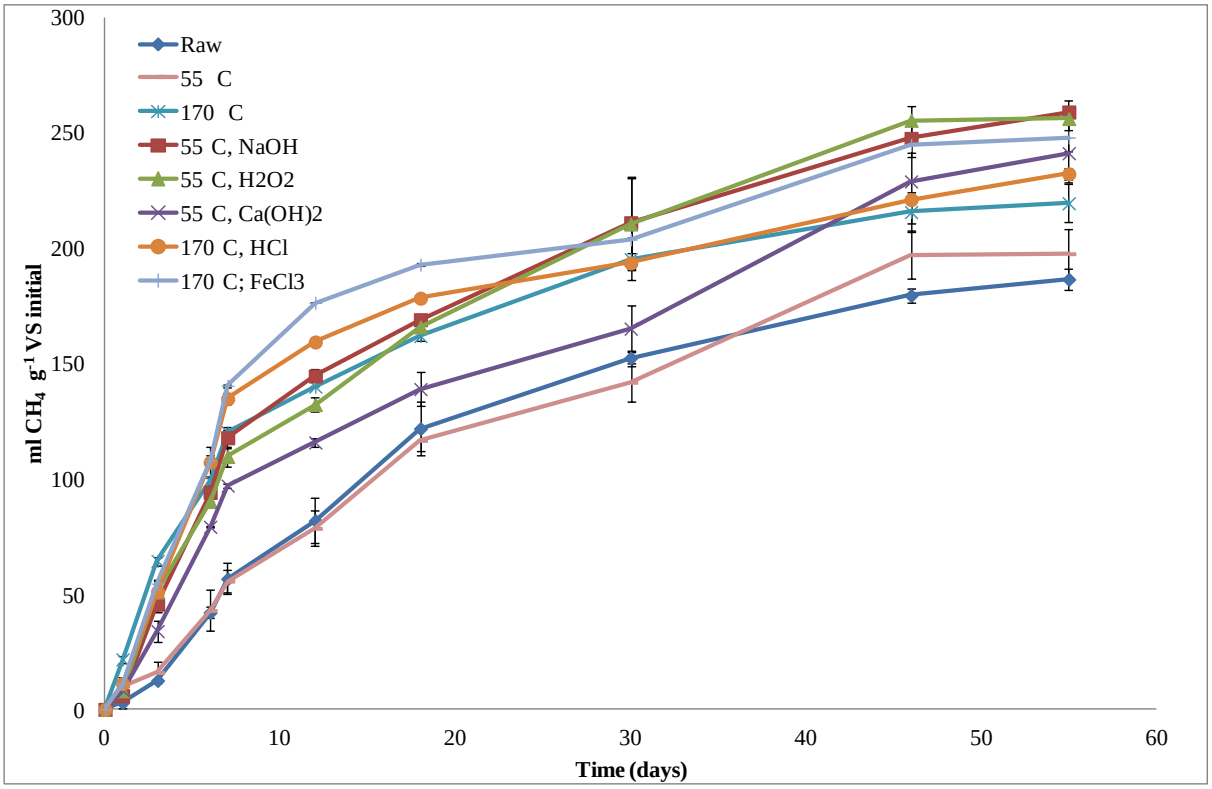

Figure VI. 1 *Methane potential curves for pretreated and untreated sunflower stalks.*

The theoretical methane potential (mL CH_4 g^{-1} VS) can be evaluated for each degradable compounds (uronic acids, hemicelluloses, cellulose and proteins) according to equation VI-1 (Frigon and Guiot, 2010; Lubken et al., 2010).

$$Y_{CH_4}^{Theoretical}\left(L/g_{substrate}\right)=\frac{22.4(4a+b-2c-3d-2e)}{8(12a+b+16c+14d+16e)} \quad \text{(Equation VI-I)}$$

Equation IV-1 leads to methane potentials of 415 mL CH_4 g^{-1} cellulose $(C_6H_{10}O_5)_n$, 424 mL CH_4 g^{-1} xylan $(C_5H_8O_4)n$, 288 mL CH_4 g^{-1} uronic acids $(C_6H_{10}O_7)$, and 420 mL CH_4 g^{-1} proteins $(C_{14}H_{12}O_7N_2)n$.

Since sunflower stalks are composed of cellulose, hemicelluloses, uronic acids and proteins (34, 20.8, 7.6, and 5.2% VS, respectively), thus a methane potential of 272 mL CH4 g^{-1}VS can be theoretically expected.

The biodegradability of substrates before and after pretreatment can be determined as follows:

BD (%) = [Experimental methane potential (mL CH4.g^{-1} VS) / 272 mL CH4.g^{-1} VS]*100 (Equation VI-II)

A methane potential of 192 mL CH_4 g^{-1} VS was observed for raw sunflower stalks that corresponded to a biodegradability of 71%, which is lower than that reported by Antonopoulou et al. (2010) on sunflower

straw with 240 mL CH_4 g^{-1} VS. For all conditions studied, except for 55°C alone, an increase in methane potential was observed, compared to raw sunflower stalks (Table VI.1).

Thermal pretreatment at 55 °C did not increase methane potential of sunflower stalks, on the contrary a slight increase of 14 % in methane potential compared to raw substrate was noticed at 170°C (219 (±8) mL CH_4 g^{-1} VS). Antonopoulou et al. (2010) showed an increase around 13% when sunflower straw was pretreated at 121 °C for 60 min. As for thermo-chemical pretreatments, an increse in methane potentials as 26, 33 % and 35 % were observed with 4% $Ca(OH)_2$, at 55°C, for 24 h; with 4% H_2O_2, at 55°C, for 24 h (pH = 11.5), and with 4% NaOH, at 55°C, for 24 h respectively. Despite the fact that no significant difference in methane potential was found after hydrogen peroxide and sodium hydroxide pretreatments, as confimed from Anova-p value (0.57), alkaline pretreatment is recommended. Indeed, for oxidative pretreatment, 3% NaOH (w/w) was added to 4 % H_2O_2 (w/w) for obtaining a pH of 11.5, because hydrogen peroxide was found more efficient to delignify lignocellulosic substrate at an optimal pH of 11.5 (Gould, 1985).

The best methane potential enhancement was observed with 4% NaOH, at 55°C, for 24 h with a methane potential of 259 (±6) mL CH_4 g^{-1} VS, corresponding to a biodegradability of 95%. However, this value should be considered as a rough estimate insofar as the lipid content was not estimated for the assessment of biodegradability. Similar results were observed after alkaline pretreatment of sorghum forage and wheat straw with 10% NaOH (w/w TS) at 40°C for 24 h as increases in the methane potential of 31 % and 47 % were achieved respectively (Sambusiti et al., 2012c). Similar trend was noticed by Zhu et al. (2010a) with an increase in methane potentials of corn stover as 40 % after alkaline pretreatments (5% NaOH, 20°C, 24 h) (Zhu et al., 2010a). Xie et al., (2011) have also noticed an increase of methane potential of 39 % on grass silage after thermo-alkaline pretreatment (100°C, 7.5 % (w/w), 48h). However, the effect of thermo-chemical (H_2SO_4 and NaOH) pretreatments on sunflower straw was investigated by Antonopoulou et al. (2010). Contrary to our results, these authors did not observe an increase in methane potential even if the solubilisation of sugars was enhanced. It is possible that the different results were caused by the differences in temperature as Antonopoulou et al. (2010) conducted pretreatment at 121°C.

Lower biodegradabilities were observed after thermal pretreatments at 170°C and with HCl as reagent (81 % and 86 %, respectively). However methane potentials were enhanced by 219 mL CH_4 g^{-1} VS (170°C, 1h) and

233 mL CH_4 g^{-1} VS (170°C, 1 h, 4 % HCl) compared to raw sunflower stalks (192 mL CH_4 g^{-1} VS). Promising results were also noticed with $FeCl_3$ pretreatments, with an increase of methane potentials by 29 % and biodegradability by 91 %.

The anaerobic digestion process was assumed to follow a first order kinetic, as it is the case of substrate where hydrolysis is the limiting steps, such as lignocellulosic residues (Angelidaki et al., 2009). For all samples, the first-order kinetic model was successful in modeling the experimental methane production during the first 18 days (R^2 > 0.94), suggesting that such a simple model is efficient to describe the complex anaerobic of lignocellulosic substrates. The first order kinetic constants k (d^{-1}) are shown in Table VI.1. For oxidative and alkaline pretreatments, small increases in kinetics constant (k) were observed: as 0.023 d^{-1} (+5 %), 0.027 d^{-1} (+23 %) and 0.028 d^{-1} (+27 %) were observed for $Ca(OH)_2$, H_2O_2 and NaOH, respectively. Similar trends were observed by Fernandes et al. (2009) which show an increase (+ 30%) in first order hydrolysis rate constant from 0.088 to 0.115 d^{-1} after alkaline pretreatment ($Ca(OH)_2$) of hay. Higher increase of first order hydrolysis rate constant of 34 % and 52 % were observed on sorghum and wheat straw after alkaline pretreatment with 10% NaOH (w/wTS) at 40 °C for 24 h and alkaline concentration (Sambusiti et al., 2012c).

Thermo-pretreatment at 170°C increased the first order kinetics constant by 64% (0.036 d^{-1}). Higher first order kinetics constants of 0.039 and 0.040 d^{-1} were recorded for acidic pretreatment (HCl and $FeCl_3$) that correspond to 77 % and 82% increases, respectively.

Table VI. 1 *Methane potential and first order kinetics constant of raw and pretreated sunflower.*

Conditions	BMP (mL CH_4 g^{-1} initial VS)	BMP increase (%)	BD[a] (%)	k (d^{-1})	k increase (%)	R^2
Raw	192 (±2)	-	71	0.022	-	0.97
24 h, 55°C	198 (±11)	3	73	0.022	0	0.99
24 h, 55°C, 4 % NaOH	259 (±6)	35	95	0.028	27	0.96
24 h, 55°C, 4 % H_20_2	256 (±2)	33	94	0.027	23	0.97
24 h, 55°C, 4 % $Ca(OH)_2$	241 (±13)	26	89	0.023	5	0.94
1h, 170°C	219 (±8)	14	81	0.036	64	0.94
1h, 170°C, 10 % $FeCl_3$	248 (±6)	29	91	0.04	82	0.95
1h, 170°C, 4 % HCl	233(±2)	21	86	0.039	77	0.96

[a] BD (%) : Biodegradability calculated taking into account the content in uronic acids, hemicelluloses, cellulose and proteins (Equation IV-2)

2.2 Correlation between biochemical and structural changes and anaerobic digestion performances

Thermal pretreatments and thermo-chemical pretreatments were found efficient to enhance methane production due to the compositional and structural changes as shown in chapter IV. In this part, we have attempted to correlate both methane production and methane production rate with chemical and structural changes induced by thermo-chemical pretreatments. In chapter III, a PLS models was previously built on a set of 18 lignocellulosic substrates to identify parameters that affect negatively and positively methane production. In was found that lignin content and, in a lesser extent, the crystallinity of cellulose have a negative impact on methane production whereas soluble carbohydrates have a positive impact. Thus the BMP of seven pretreated samples (two thermal (55°C, 170°C) and five thermo-chemical pretreatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) was predicted using the PLS model determined in chapter III. For the soluble carbohydrates concentration, the sum of solubilized cellulose, uronic acids and hemicelluloses was considered. Interestingly, the PLS model was found efficient in predicting the methane potentials of the seven pretreated samples as they were found under the uncertainty limit of the model with an error between methane potentials determined by the model and by experiment lower than 6.5 % (Figure VI.2).

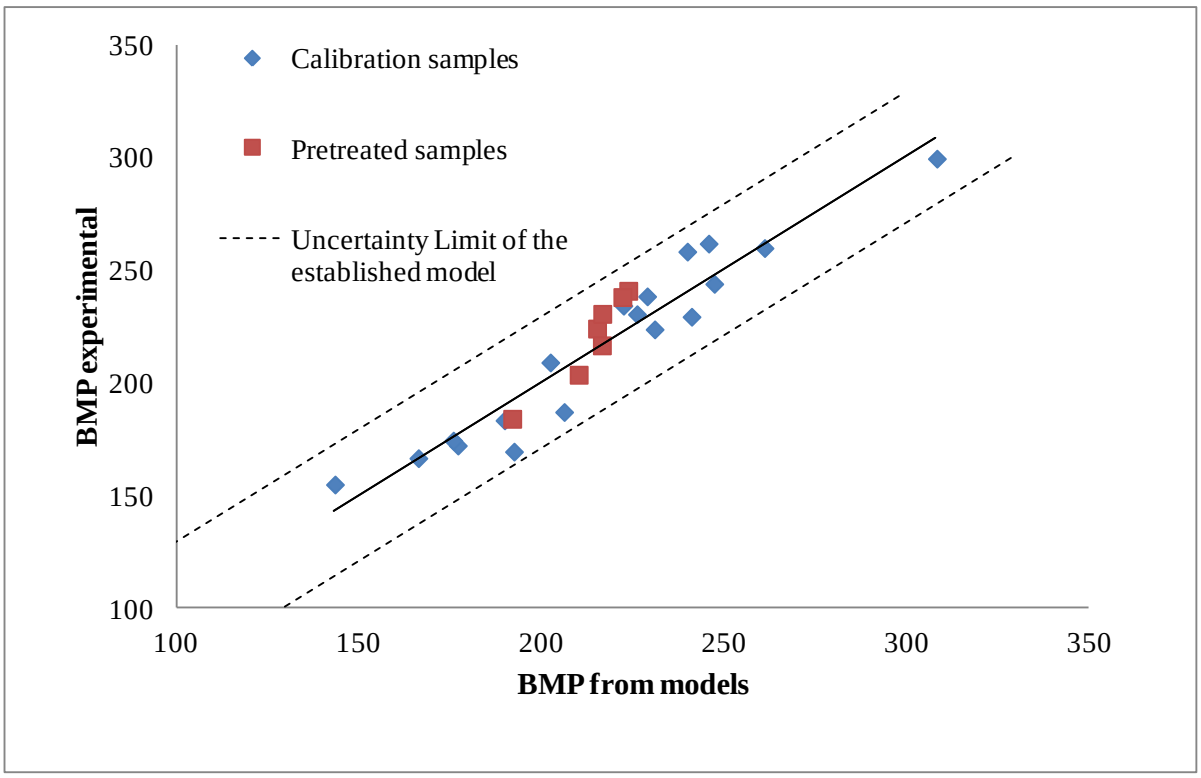

Figure VI. 2 *Experimental methane potential vs predicted methane potential (according equation III-2 of chapter III).*

Thus, this model seems to be appropriate to explain the variation of methane potentials observed during thermal and thermo-chemical pretreatments of the sunflower stalks. Lignin content seems to be the major parameter affecting methane potentials of sunflower untreated and pretreated. Indeed, a strong negative correlation (R^2 = 0.91) was found between the methane and the lignin content (Figure VI.3a). These results were also observed by Kobayashi et al. (2004) with steam-exploded bamboo where a linear regression showed a strong negative correlation (R^2 = 0.95) between the amount of methane produced and the amount of lignin. Similar strong negative correlation (R^2 = 0.98) was also observed between the amount of Klason lignin in steam-exploded wood chips and the amount of methane gas produced (Take et al., 2006). Triolo et al. (2011) also found a good negative correlation (R^2 = 0.88) between lignin content and methane potentials of energy crops and manure. Like previously mentioned in chapter III, the lignin content plays a major role in methane production by limiting access of microorganisms to holocelluloses. Moreover, due to its non-water-soluble nature, lignin represents the most recalcitrant part of the plant structure (Nizami et al., 2009). The holocelluloses (hemicelluloses and cellulose), which are anaerobically-biodegradable in their pure form, appear to be less biodegradable or even completely refractory when combined with lignin (Tong et al., 1990). According to Zhu et al. (2010b), removing lignin enables a high rate of biomass conversion. Moreover, the presence of furfural and 5-HMF quantified in chapter IV during thermal pretreatment (170°C), with or without acid reagents, did not seem to affect neither the methane potentials nor the methane production rate, possibly due to the low concentrations observed and to the dilution of samples in BMP tests. Such results are in agreement with Barakat et al. (2012) that showed that such byproducts (furans derivatives and phenolic compounds) added separately at the same concentration (1 g L^{-1}) were not inhibitory of methane production from xylose.

Moreover, a good correlation (R^2 = 0.91) was found between the first order kinetic constant and the sum of solubilised proteins, hemicelluloses, cellulose and uronic acids (Figure VI.3b). Consequently, soluble matter originating from the removal of protein, hemicelluloses, cellulose and uronic acids plays an important role in the rate of anaerobic digestion of pretreatred lignocellulosic residues; the greater the soluble matter, the higher the kinetic constant. Contrary to Zhu et al. (2010b) who showed that decreasing crystallinity accelerates hydrolytic reaction in the biomass, no significant correlation was found between

crystalline cellulose removal and the first order kinetic constant, perhaps due to the low variation range of crystalline cellulose (16 to 19.3 % initial VS showed in Table IV.2 of the chapter IV) in our study.

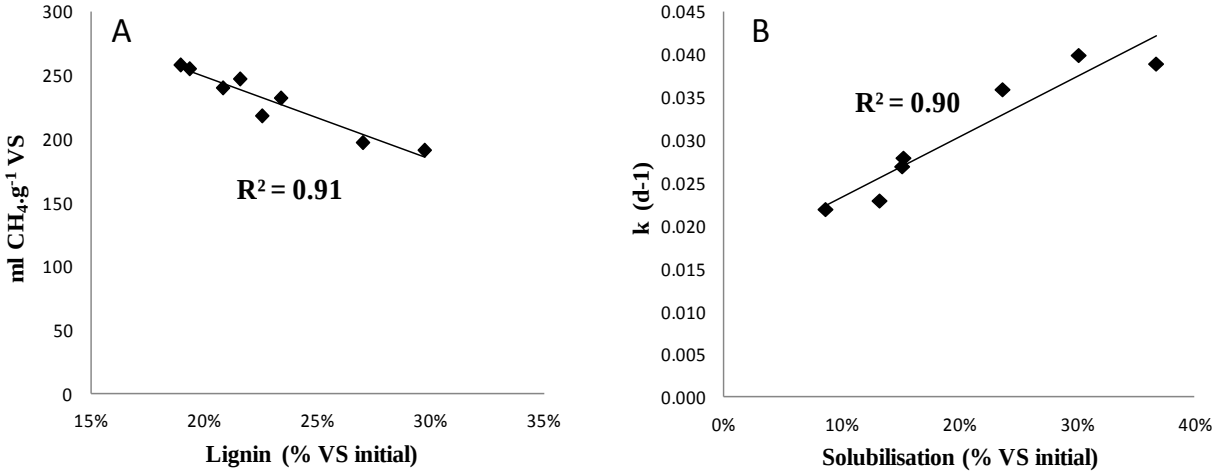

Figure VI. 3 *Correlation between methane potential and the lignin content of raw sunflower stalks and residual solid fraction of pretreated sunflower stalks (a); correlation between the first order kinetic constant and the sum of solubilisation of proteins, cellulose, hemicelluloses and uronic acids of pretreated sunflower stalks (b)*

3. Impact of alkaline pretreaments parameters on methane production of sunflower stalks

Alkaline pretreatments using sodium hydroxide was previously found as the best pretreatment to enhance methane from sunflower stalks with an increase by 35%. Thus, this pretreatment was selected to study the impact of operating parameters (time, temperature, acid concentration) variations on the methane potentials (Figure VI-4).

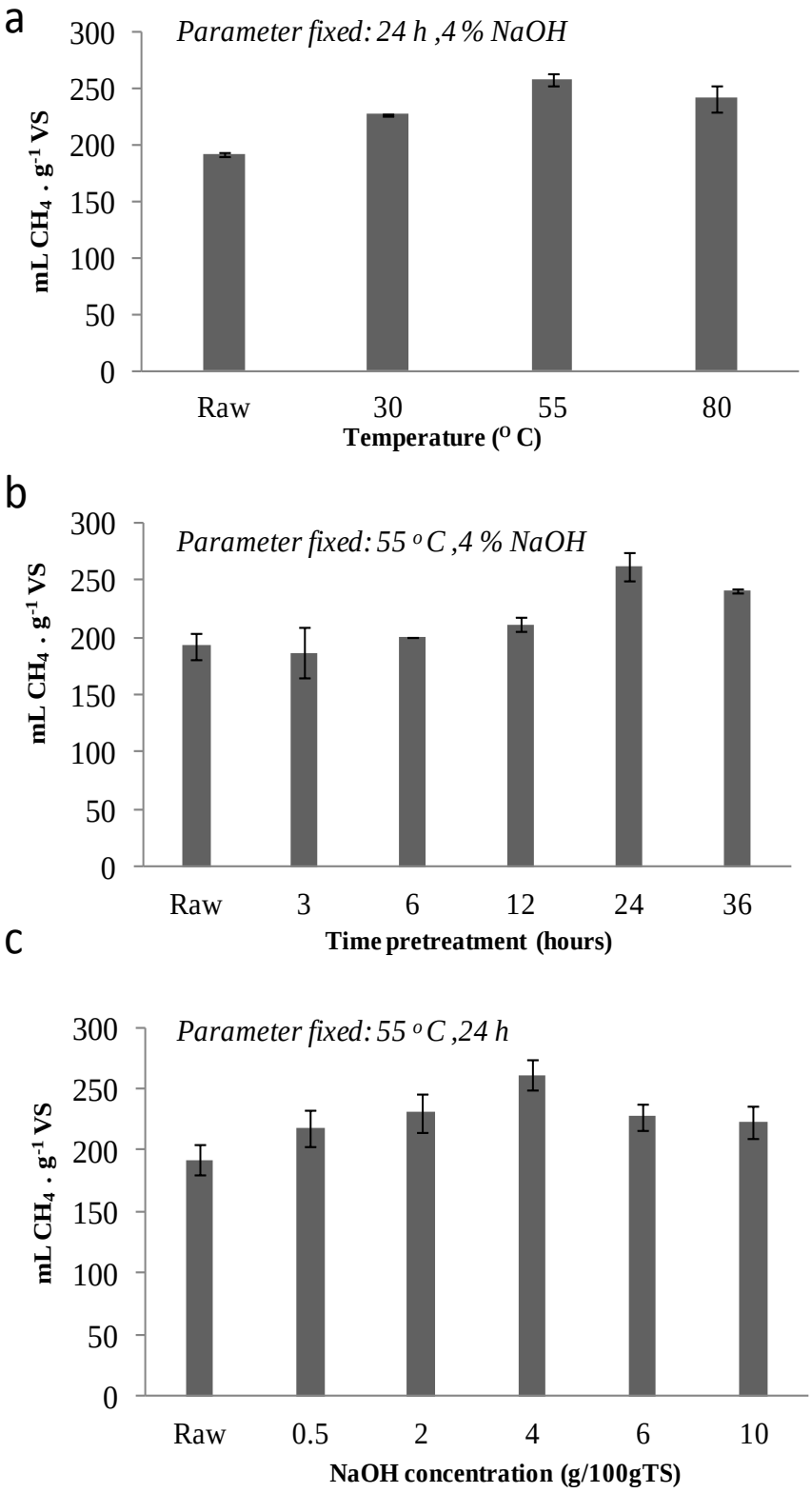

Figure VI. 4 *Optimization of alkaline pretreatments parameters: (a) temperature, (b) time and (c) NaOH concentration.*

Temperature parameter was first investigated on "NK-Kondi" sunflower stalks with three temperatures (30°C, 55°C and 80°C) at fixed time (24 h) and fixed alkaline concentration (4% NaOH (w/w)). For the three temperatures (30°C, 55°C and 80°C), methane potentials increased significantly compared to raw "NK-Kondi" (192 (±2) mL $CH_4.g^{-1}$ VS). Best pretreatments temperature was 55°C with an increase of methane potential of 35 % (Figure VI.4a). The analysis of variance (Anova) method with 95% confidence level showed a significant difference between 30 °C and 55°C pretreatments with Anova p-value of 0.015 (<0.05) but no significant difference was noticed between 55°C and 80°C (p=0.19). As performing pretreatment at 55°C is less expensive than 80°C, temperature of 55°C was selected for further experiment.

Then, different pretreatment times (3 h, 6 h, 12 h, 24 h and 36 h) were investigated at fixed temperature (55°C, optimal temperature from the previous analysis) and fixed alkaline concentration (4% NaOH (w/w) on "Serin" sunflower stalks. For pretreatment times less than 24 h, i.e. 3 h, 6 h and 12 h, no significant increase of methane potentials compared to raw substrate was noticed (Figure VI.4b). Higher methane potential was observed after 24 hours with methane potential of 262 (±12) mL CH_4 g^{-1} VS corresponding to an increase of 36 % compared to raw sunflower stalks. Moreover significant differences were noticed between 12 h and 24 h as Anova p-values of 0.035 was obtained but not between 24 h and 36h (p=0.14). Like previously, less expensive pretreatment, 24 h rather than 36 h, was selected as fixed variable for the last experiment series where alkaline concentration variations were investigated.

Indeed, alkaline concentrations (0.5 %, 2 %, 4%, 6% and 10 % NaOH (w/w)) were studied at fixed temperature (55°C) and fixed time (24 h) on "Serin" sunflower stalks (Figure VI.4c). According Anova p-value, no significant differences were observed between the different alkaline concentrations (0.5 %, 2 %, 4% 6% and 10 % NaOH) maybe due to the lack of replicates (only duplicates) in the experiment. Such results are in disagreement with Xie et al., (2011) who observed significant methane potentials increase on grass silage by increasing NaOH concentrations from 1% to 7.5% (w/w) at 100°C for 48 h.

However, only the alkaline concentration of 4 % was found to have a significant impact (Anova p-value = 0.04) with an increase of methane production from 262 (±12) mL CH_4 g^{-1} VS compared to 193 (±16) mL CH_4 g^{-1} VS for raw sunflower stalks. To conclude, according to these previous results, the optimal alkaline

pretreatment conditions were 55°C and 24 h and 4% NaOH These conditions was selected for further application in continuous reactors.

4. Application of the best pretreatment conditions to other sunflower stalks.

In this part, the best alkaline pretreatments conditions previously defined (55°C, 24 h, 4% NaOH) were applied on four different sunflower stalks samples (NK-Kondi, Naturasol and Serin1 and 2). Both Serin sunflower stalks were not grown at the same place and above all their storage conditions were different. Serin 1 stalks were collected in the field two weeks after harvest whereas Serin 2 stalks were collected around two months after the harvest. One objective of this sub-chapter was to show if the previous benefit observed in term of methane potential on sunflower stalks can be observed on other varieties with different but relatively closed biochemical compositions. To achieve this target, the effect of alkaline pretreatment (55°C, 24 h, 4% NaOH) on biochemical composition and methane production were compared for the four sunflower stalks.

Biochemical composition in terms of uronic acids, cellulose, hemicelluloses and lignin content of the four sunflower stalks is shown in table VI.2.

Table VI. 2 *Biochemical composition of four sunflower stalks in % of VS. Values correspond to means of two replicates of independent values ± standard deviation.*

	Uronic acids	Cellulose	Hemicelluloses	Lignin
Serin 1	2.2 (± 0.3)	25.1 (± 1.7)	11.5 (± 1.2)	32.5 (± 0.7)
Serin 2	2.2 (± 0.4)	20.8 (± 0.1)	9.2 (± 0.1)	25.05 (± 1)
Naturasol	1.3 (± 0.3)	23.3 (± 0.9)	9.4 (± 1)	33.5 (± 2.2)
NK-Kondi	7.6 (± 0.3)	34.03 (± 0.5)	20.8 (± 0.8)	29.7 (± 0.6)

The sum of these four compounds (uronic acids, cellulose, hemicelluloses and lignin) accounted for 57.3 % to 92.16 % of the total VS suggesting that part of the matter was not quantified (ie lipids, proteins or others sugars) or that external microorganisms or fungi were present in the sunflower stalks and interfered in the determination of VS.

Among biochemical composition, some differences were noticed between the four sunflower stalks. Indeed, uronic acids contents of 1.3 %, 2.2 %, 2.2 % and 7.6 % VS were respectively observed for Naturasol, Serin 1, Serin 2 and NK-Kondi. Holocelluloses contents varied between 30 % - 37 % except for NK-Kondi sunflower stalks which had a higher holocelluloses content of 55 % VS. Finally lignin contents varied from 25 % VS (Serin 2) to 33.5 % VS (Naturasol). High difference in lignin content was found between the same variety Serin 1 and Serin 2. One explanation can be that Serin 2 was let during a higher time in the field and thus was exposed to some fungi and microorganisms present in the soil and which are able to degrade lignin (Tuomela et al., 2002, Dashtban et al., 2009, Saritha et al., 2012).

Table VI. 3 *Loss percentages based on % VS for uronic acids, cellulose, hemicelluloses and lignin for the four sunflower stalks.*

	Uronic acids	Cellulose	Hemicelluloses	Lignin
Serin 1	61.3%	3.4%	23.2%	23.3%
Serin 2	55.2%	2.0%	8.0%	26.4%
Naturasol	0.0%	16.9%	16.7%	33.0%
NK-Kondi	71.7%	0.0%	26.0%	36.3%

In Table VI.3, loss percentages for uronic acids, cellulose, hemicelluloses and lignin after alkaline pretreatment for the four sunflower stalks are presented. Except for Naturasol sunflower stalks, alkaline pretreatment was found efficient in uronic acids removals with reduction yields higher than 55 %. For each sunflower stalks, low or no cellulose removals were noticed. Partial hemicelluloses removals varying from 8% (Naturasol) to 26% (NK-Kondi) were noticed. In conclusion, alkaline pretreatment was found efficient to remove part of the lignin with reduction yield ranged from 23.3% (Serin 1) to 36.3% (NK-Kondi). Such results are in accordance with previous results presented in chapter IV, and from several literature studies that highlighted that alkaline pretreatment are efficient to delignify and remove part of hemicelluloses (Taherzadeh and Karimi, 2008, Xie et al., 2011).Then, batch anaerobic assays were carried out on the four varieties of sunflower stalks with or without alkaline pretreatment. Results of methane potentials are presented in Figure VI.5.

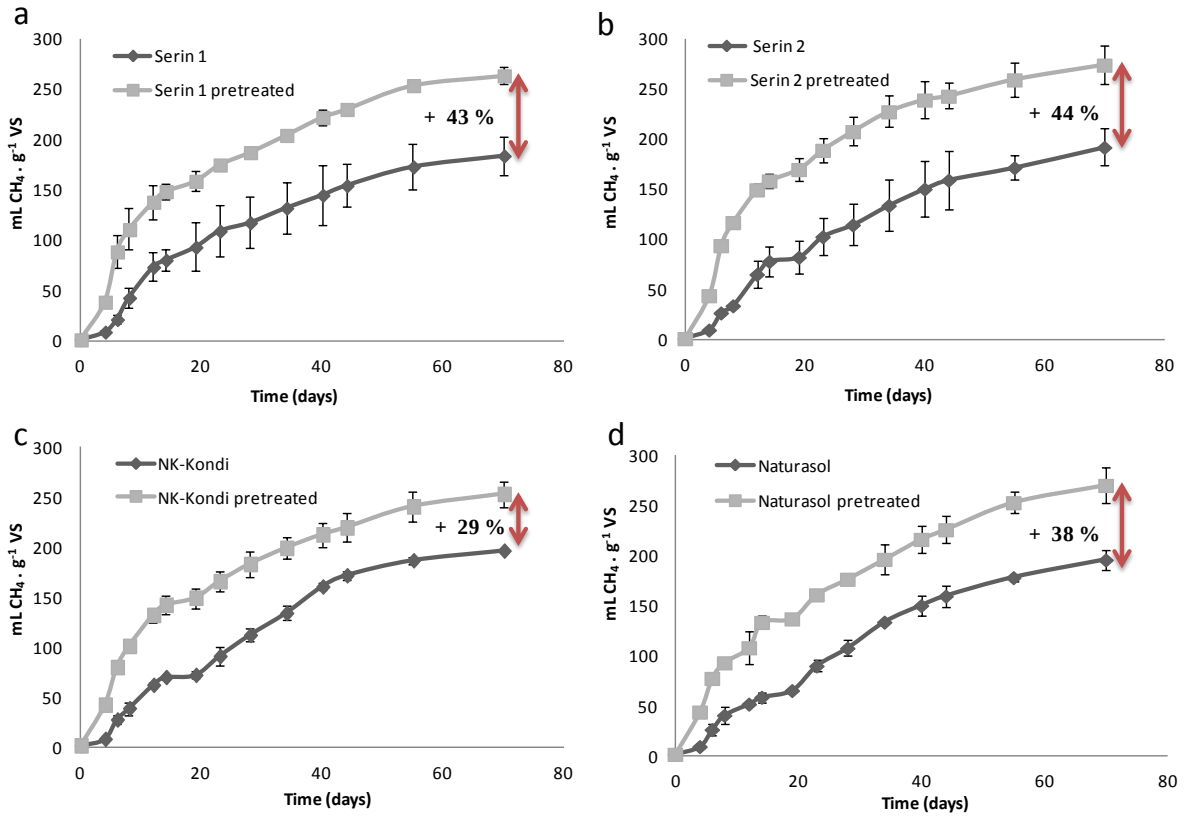

Figure VI. 5 *Methane potentials (mL CH_4. g^{-1} VS initial) from untreated and alkaline (55°C, 24 h, 4% NaOH) pretreated sunflower stalks for the following varieties: (a) Serin 1, (b) Serin 2, (c) NK-Kondi and (d) Naturasol.*

Methane potentials of 183, 184, 197 and 206 mL CH_4 g^{-1} VS were respectively observed for Naturasol, Serin 1, NK-Kondi and Serin 2. Alkaline pretreatment was shown efficient to enhance methane potentials with increases of 29%, 38%, 43% and 44% respectively for NK-Kondi, Naturasol, Serin 1 and Serin 2. Such results are interesting as they suggest that alkaline pretreatment can be applied to enhance significantly methane potentials from various varieties of sunflower stalks. However it should be interesting to extent such observations to substrates with biochemical and structural characteristics highly different (ie, grasses, energy crops, hardwood, softwood etc.). Indeed, Fernandes et al. (2009) have shown that calcium hydroxide pretreatment was efficient to enhance methane production of bracken (+142%) but not of straw (0%) and hay (-12.5%). Efficiency of pretreatment seems to be highly dependent on both content and structure of the lignocellulosic biomass (Wyman et al., 2005).

5. Conclusions

A comparative investigation of two thermal (55°C and 170°C) and five thermo-chemical pretretatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) was carried out on methane potentials of "NK-Kondi" sunflower stalks. The highest methane production (259 ± 6 mL $CH_4.g^{-1}$ VS) corresponding to an increase of 35 % was achieved after alkaline pretreatment. Moreover, it was shown that methane production rate was positively correlated to the amount of solubilized matter whereas methane potential was negatively correlated to the amount of lignin which is an agreement with previous observation of chapter III. In a second part, the impact of alkaline pretreatment conditions (temperature, time, and concentration) was investigated to enhance methane potentials of sunflower stalks and an optimal condition was found to be: 55°C, 24 h, 4% NaOH. This optimal condition was further applied on four sunflower stalks (Naturasol, Serin 1, NK-Kondi and Serin 2) and methane potentials increases of 29 %, 38 %, 43 % and 44 % were respectively observed for NK-Kondi, Naturasol, Serin 1 and Serin 2. Thus, alkaline pretreatment was found efficient to enhance methane production of sunflower stalks with different but relatively closed chemical composition. However, such results should be extended to other substrates with highly different biochemical and structural characteristics for further industrial application. Indeed, at industrial scale, fermenters are fed either with rotations of lignocellulosic substrate or ensiling substrate presenting different biochemical composition. Indeed, silage process is known to induce changes in the biochemical composition of substrates (Herrmann et al., 2011). Moreover, previous observations on enhancement of methane production using alkaline pretreatment should be applied in continuous reactor to be close to full scale anaerobic digestion plant and establish economic and energetic balances.

Chapter VII. Continuous anaerobic digesters: performances, energetic and economic aspects

1. Introduction

In this chapter of the thesis, two main objectives were considered. The first one was to validate, in continuous anaerobic digester, previous results obtained in batch mode. Since methane production is nowdays far more developed at industrial scale than hydrogen production, this chapter focuses on enhancing methane production using the best pretreatment conditions (4% NaOH, 55°C, 24 h) previously found in batch anaerobic conditions. For this, raw sunflower stalk was compared to pretreated sunflower stalks as feeds of continuous anaerobic reactors (configuration 1 and 2 in Figure VII.1).

In a second objective, the impact of two-stage H_2 (batch)/CH_4 (continuous) process compared to one-stage CH_4 process was investigated. Indeed, the two-stage H_2/CH_4 process appears to be a promising way to use the outlet of dark fermentation bioprocess rich in metabolites, mainly acetate and butyrate. Moreover a two-stage H_2/CH_4 process permits to produce a "biohythane" gas which is described having a more stable combustion than methane and in the same time lowering exhaust gas emissions (Sierens and Rosseel, 2000). To date, only few studies have investigated the benefit of two-stage processes H_2/CH_4 compared to one-stage CH_4 and most of these studies have been performed in batch mode (Pakarinen et al., 2009; Pakarinen et al., 2011; Cheng and Liu., 2011b; Kongjan et al., 2010).

In the case of configuration 3, H_2 stage was performed in mesophilic batch assay due to the difference in hydraulic retention times, ie generally few hours for H_2 stage and several days for anaerobic digesters. Considering an anaerobic digester with a working volume of 1.5 L and an HRT of 21 days, if the HRT of H_2 was fixed at 12 hours, the working volume of the H_2 stage would have been of 36 mL. As it was difficult to consider such a small H_2 reactor at the laboratory scale, H_2 stage was performed in mesophilic batch.

Three anaerobic mesophilic digesters were carried out with identical reactors with a working volume of 1.5 L, a HRT of 21 days and a OLR of 1.49 gVS L^{-1} d^{-1} (Figure VII.1). In configurations 1, 2 and 3, anaerobic digester were fed with raw "Serin" sunflower stalks, pretreated (4% NaOH, 55°C, 24 h) sunflower stalks and with the outlet of H_2 batch stage of raw "Serin" sunflower stalks , respectively.

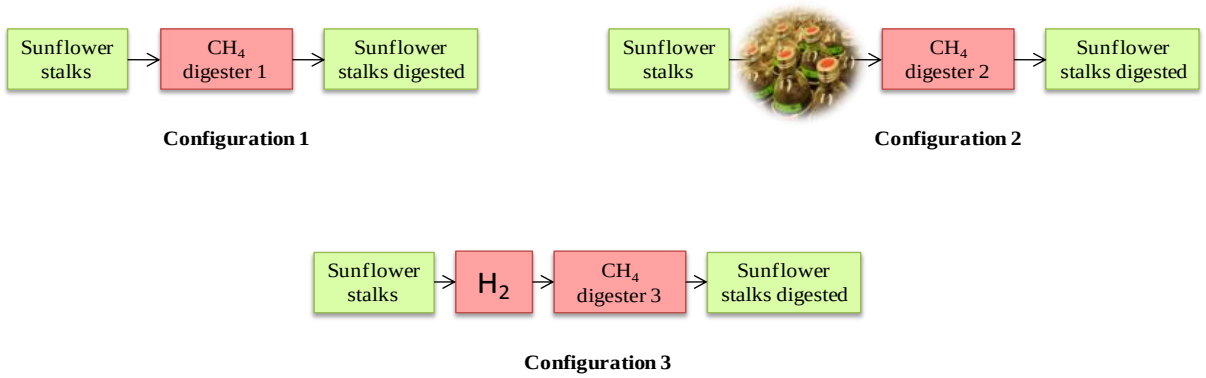

Figure VII. 1 *Different configurations of continuous anaerobic digesters.*

In the first part of this chapter, configurations 2 and 3 were compared to configuration 1 in terms of methanogenic performances: total energy production, TS and VS removals. In a second part, the energetic and economic aspects were investigated for the three configurations to determine the feasibility of such processes at industrial scale.

2. Digesters performances

First anaerobic performances of configurations 2 and 3 were compared with configuration 1 fed with raw "Serin" sunflower stalks in terms of total solids (TS) removal, volatile solids (VS) removal and biogas production. Methane production for the three anaerobic digesters during the three hydraulic retention times (9 weeks) is presented in Figure VII.2. After the first two hydraulic retention times (week 6), the anaerobic digesters were considered at steady state. Thus, in the rest of this chapter performances of the three anaerobic digesters will be discussed on the results of the third hydraulic retention time (weeks 7-9). Main results are presented in Table VII.1.

Table VII. 1 *Digesters performances for the three configurations during the 3rd HRT measured over two days for methane production and over weeks for the other parameters*

	Configuration 1 (one-stage CH_4)	Configuration 2 (NaOH one-stage CH_4)	Configuration 3 (two stages H_2/CH_4)
Feedstock parameters			
TS (g L^{-1})	33,6	35	33,6
VS (g L^{-1})	31,1	31,1	31,1
pH	8	11,1	6[a]
H_2 batch assay			
H_2 production (NmL g^{-1} VS added)			7.1
Acetate (g L^{-1})			0.55
Butyrate (g L^{-1})			0.40
pH outlet			5.5
CH_4 continuous reactor	Digester 1	Digester 2	Digester 3
pH outlet	6.6 (±0.1)	6.9 (±0.05)	6.9(±0.1)
TS (g L^{-1}) outlet	22.4 (±0.1)	20.6 (±0.2)	22.8 (±0.0)
VS (g L^{-1}) outlet	18.9 (±0.4)	16.3 (±0.2)	18.9 (±0.0)
VS/TS	0.84	0.79	0.83
Biogas production (mL d^{-1})	646 (±10)	782 (±14)	603 (±15)
CH_4 (%)	52.5 (±2)	54.6 (±2)	56 (±3)
CH_4 production (NmL g^{-1} VS added)	152 (±4)	191 (±3)	150 (±3.5)
Increase of CH_4 production (%)		25.6	-1.3
Total Energy (kWh t^{-1} VS)[c]	1520 (±40)	1910 (±30)	1506 (±38)
Increase of Total Energy (%)		25.6	-1
Removal			
Removal TS (%)	33	41	32
Removal VS (%)	39	48	39
Increase of TS removal[b]		23	-3
Increase of VS removal[b]		21	0

[a] Before dark fermentation stage the pH was adjusted to 6
[b] Increase is expressed as percentage over digester 1
[c] Methane conversion was considered as 10 kWh m^{-3} and hydrogen conversion was considered as 3 kWh m^{-3}

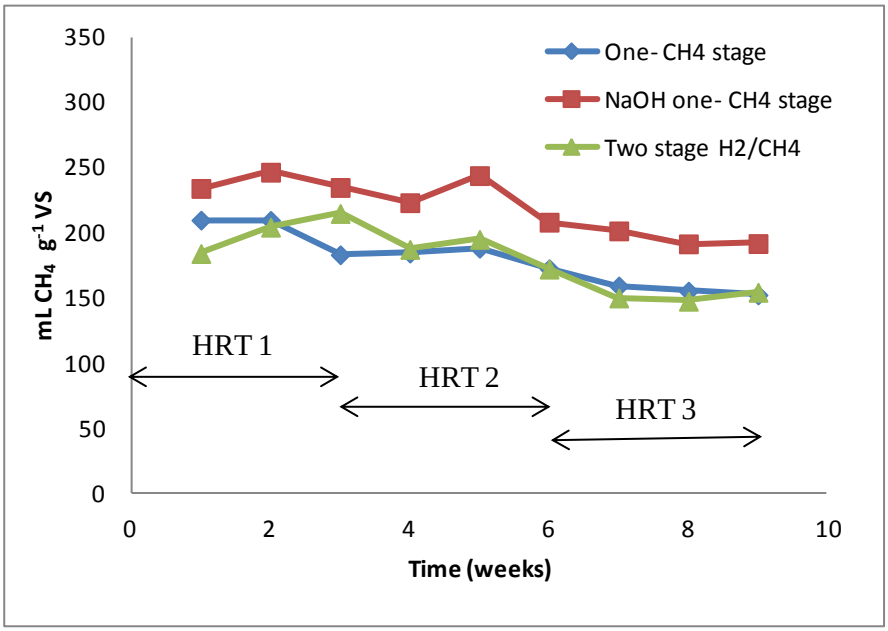

Figure VII. 2 *Variation of methane production for the three anaerobic digesters during the three hydraulic retention times.*

2.1 Effect of alkaline pretreatment on one-stage CH_4 process

TS and VS removals in digesters outlets 1 and 2 were considered at pseudo steady state (3rd hydraulic retention times, ie weeks 7 to 9). A reduction of 33 % and 39 % were respectively observed for TS and VS removals in the digester 1 fed with raw sunflower stalks. Alkaline pretreatments (4% NaOH, 55°C, 24 h) enhanced TS and VS removals up to 41% and 48 % for TS and VS in digester 2 (Table VII.1). The decrease of the ratio VS/TS from 0.84 to 0.79 between configuration 1 and 2 in the digestate is probably due to the substancial addition of sodium hydroxide in digester 2.

In digester 2, it was showed that the use of sodium hydroxide during the pretreatment step was not detrimental to the anaerobic process due to the low concentration used (4% NaOH (w/w)) equivalent to 0.8 g Na^+. L^{-1}. Such concentration remains much lower than 4.1 g Na^+ L^{-1} which was found to be an inhibitory concentration from methane production with seaweed (Jard et al., 2012). In addition, due to a dilution effect, production of VFAs and the subsequent carbonate buffer effect in the reactor, the pH at the end of digester 2 was neutral (pH =6.9) and very close to that observed in digester 1 (pH =6.6), with no regulation in both systems.

In term of methane production, alkaline pretreatment (4% NaOH, 55°C, 24 h) was found highly beneficial as 191 (±3) mL CH_4 g^{-1} VS initial were produced from digester 2 compared to 152 (±4) mL CH_4 g^{-1} VS initial in digester 1 which corresponds to an increase of 25.6 % in term of methane yield.

Moreover, a VS recovery check was computed for all reactor configurations, by considering "Sunflower stalks" as imput variable and "methane", "carbon dioxide", "hydrogen" and "digestate" as output variables. Satisfactory results of recovery were obtained for configuration 1 and 2 supporting the reliability of reactors results (Table VII.2).

Table VII. 2 *VS balances for the three configurations*

	Configuration 1 (CH_4)	**Configuration 2 ($NaOH$-CH_4)**	**Configuration 3 (H_2/CH_4)**
Sunflower stalks (kg VS/tonTS)	890	890	890
Methane (kg VS/tonTS)[a]	97	122	96
Carbon dioxide (kg VS/tonTS)[a]	241	279	212
Hydrogen (kg VS/tonTS)[a]			0,2
Digestate (kg VS/tonTS)	543	462,8	543
Relative difference balances (%)[b]	1%	3%	4%

[a] It is assumed that 16 g CH_4, 44 g CO_2 and 2g H_2 is equivalent to 22.4 L in conditions standard of temperature and pression (0°C, 1.013 hPa).
[b] Balances = [sunflower stalks-(methane+hydrogen+carbon dioxide+digestate)/sunflower stalks]*100

2.2 Two-stage H_2/CH_4 process versus one stage CH_4

Additionally, in configuration 3, hydrogen production was considered. Results are presented in Table VII.2. The produced biogas was composed of H_2 and CO_2 only, and was free of CH_4. Hydrogen yield of 7, 1 mL H_2 g^{-1} VS was observed for untreated sunflower stalks. Initially, the pH was adjusted manually to 6 and the low decrease of the pH from 6 to 5.5 can be explained by the accumulation of metabolites (acetate and butyrate) generated by dark fermentation step. Among the metabolites found at the end of the dark fermentation stage, only acetate (0.55 g L^{-1}) and butyrate (0.40 g L^{-1}) were found indicating that hydrogen was mainly produced from the acetate-butyrate fermentation pathways.

The outlet of the dark fermentation stage with untreated sunflower stalks was further used to produce methane in anaerobic digester 3 in a two-stage H_2/CH_4 process (configuration 3). Coupling H_2/CH_4 process did not enhance significantly TS and VS removals compared to one-stage CH_4 (digester 1).

Indeed, 32% and 39% of TS and VS removals were respectively observed in digester 3 compared to the 33% and 39% observed in digester 1 (Table VII.1).

Moreover, no significant methane yield increase was observed between digester 3 (150 (±3.5) mL CH_4 g^{-1} VS) and digester 1 (152 (±4) mL CH_4 g^{-1} VS) suggesting that the two-stage H_2/CH_4 process was not efficient compared to one-stage CH_4. In term of energy, no significant difference was observed with respectively 1520 (±40) and 1506 (±28) kWh t^{-1} VS produced respectively for one-stage and two-stages. In the two-stage process, only 1.4% of the total energy was coming from the hydrogen production.

Similar trend was observed by Pakarinen et al., (2011) who carried out a two-stage H_2/CH_4 process on maize. Besides a H_2 production of 5.6 (±0.7) mL H_2 g^{-1} VS, no significant enhancement of methane potentials from 321 (±23) mL CH_4 g^{-1} VS (one-stage CH_4) to 342 (±8) mL CH_4 g^{-1} VS was observed by the authors. In another study, Pakarinen et al. (2009) showed that besides a H_2 production of 5.6 (±0.6) mL H_2 g^{-1} VS, a two-stage H_2/CH_4 process can increase slightly the methane production from grass silage compared to a one-stage process with methane potentials of 467 (±18) mL CH_4 g^{-1} VS and 431 (±3) mL CH_4 g^{-1} VS respectively. However, such comparison should be carefully considered as substrates used were different from our study. Moreover, methanogenic processes, were performed in batch conditions whereas in continuous mode in our study.

The absence of increase in methane production in two-stage H_2/CH_4 can be explained by the low performances observed during the H_2 stage that did not permit to enhance CH_4 production. Indeed, enhancement of the methane yield in the two-stage process is concomittant to the fact that the H_2 production stage enhanced hydrolysis of the solid substrates and resulted in increased solubilization and VFA production (Pakarinen et al., 2009). It has been previously shown in chapter III, that hydrogen production is highly dependent on the amount of soluble carbohydrates. And yet, due to the low soluble carbohydrates content on sunflower stalks, a very small amount of metabolites (acetate, butyrate) and H_2 were produced for further enhance methane production due to its recalcitrant structure (high lignin content and low soluble sugars content). Moreover, satisfactory results of 96 %

of recovery were obtained for configuration 3 supporting the reliability of reactors results (Table VII.2).

3. Energetic and cost aspects

Since, no significant difference was shown between the total energy produced in the one stage CH_4 (1520 (±40) kWh t^{-1} VS) and two-stage H_2/CH_4 (1506 (±38) kWh t^{-1} VS) processes; the energetic and economic aspects were only considered in one stage CH_4 process versus one stage CH_4 process combined with alkaline pretreatment.

In this part, the energetic aspects will be first discussed (Table VII.3). Conversions into heat and electricity using combined heat and power (CHP) system was considered as alkaline pretreatment requires heat energy. Moreover this point is consistent with the present development of farm anaerobic digestion in European countries where biogas is converted by CHP in most of the cases. Thus, the assessment of the hydrogen and methane conversion into heat and electricity by CHP for the two configurations was achieved considering a thermal efficiency of 50 % and an electricity efficiency of 35 % (Table VII.3).

First, alkaline pretreatment was found efficient to enhance significantly heat and electricity production for configuration 2 compared to configuration 1. Indeed, heat and electricity increases of 185 KWh t^{-1} TS and 129 KWh t^{-1} TS were respectively observed for configuration 2 compared to configuration 1. Nonetheless, it is important to verify if the supplementary of heat is sufficient for the heat requirement to perform the alkaline-pretreatment. Heat requirement for alkaline pretreatment was assessed by the energy needs to raise the temperature of sunflower stalks from 25°C to 55°C assuming that specific heat of the substrate suspension in water can be evaluated by the water specific heat (4.18 kJ kg^{-1} $°C^{-1}$) as previously mentioned in the chapter Materials and Methods. This energy needed to treat 1 ton of total solids is thus highly dependent on the solid loading during pretreatment. In our study, low solid loading of 35 kg TS m^{-3} was investigated. Several studies have investigated the feasibility of applying pretreatments at higher solid loading (≥ 15 % solids, w/w) (Modenbach and Nokes, (2012). Current technologies allow the use of up to 30% solids content in starch fermentation process whereas only 15-

20% solids in lignocelluloses conversion was applied at pilot-plant scale (Modenbach and Nokes, (2012). For instance, Scheell et al. (2003) demonstrated the feasibility of a pilot scale system (1 ton day^{-1}) capable to continuously perform acid pretreatment on corn stover at 20 % solid loading (ie 200 kg TS m^{-3}). In addition, thermal energy integration has to be carried out in a full scale implementation of thermal or thermo-chemical pretreatment. This can be achieved by exploiting the high temperature and enthalphy of pretreated biomass, exhaust gases and hot water from the gas engine (Fdz-Polanco and al., 2008). The heat energy of pretreated substrate suspension can be recovered to heat the digester (Zabranska et al., 2006) or to preheat raw substrate suspension (Bougrier et al., 2007). Dhar et al. (2012) reported up to 80 % of heat recovery from thermally pretreated sludge.

Thus, to assess the interest of pretreatment, net heat production of configuration 2 was calculated as the difference between the supplementary heat produced by the conversion of methane in configuration 2 compared to configuration 1 and the heat required by the pretreatment step. To evaluate net heat production several cases were considered: a) alkaline pretreatment performed at a solid loading of 35 kg TS m^{-3}; b) alkaline pretreatment performed at a solid loading of 200 kg TS m^{-3} with the assumption that performances are the same than 35 kg TS.m^{-3}; c) alkaline pretreatment performed at a solid loading of 35 kg TS m^{-3} with 80% of the heat recovery; d) alkaline pretreatment performed at a solid loading of 300 kg TS m^{-3} with 80% of the heat recovery. Results of net heat production are presented in Figure VII.3 and expressed in kWh t^{-1} TS.

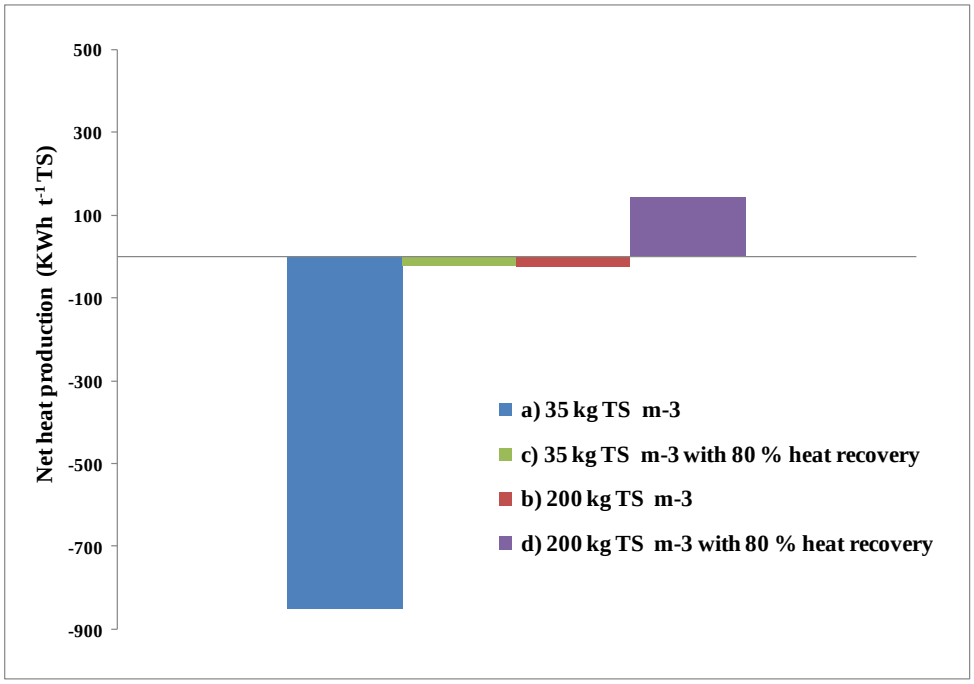

Figure VII. 3 *Net heat production (kWh t^{-1} TS) for the configuration 2 compared to configuration 1 according the solid loading of pretreatments (35 and 200 kg TS m^{-3}) with or without 80 % heat recovery*

When alkaline pretreatment was performed at a low solid loading of 35 kg TS m^{-3}, the supplementary heat produced through configuration 2 compared to configuration 1 was not sufficient to cover the heat required by the pretreatment and a heat shortage of 849 kWh t^{-1} TS was evaluated. When 80% of heat recovery was considered, the heat produced in configuration 2 was almost sufficient to cover the heat required by pretreatment as a heat shortage of 22 kWh t^{-1} TS was evaluated.

When alkaline pretreatment was performed at a high solid loading of 200 kg TS m^{-3}, the supplementary heat produced in configuration 2 was almost sufficient to cover the heat required by pretreatment as only a heat shortage of 25 kWh t^{-1} TS was observed. Finally, considering a solid loading of 200 kg TS m^{-3} with 80 % of heat recovery from the pretreatment step, net heat production was positive suggesting that the supplementary heat produced through configuration 2 was enough to cover the heat pretreatment requirement and an extra production of heat was generated (143 kWh t^{-1} TS) compared to configuration 1.

Table VII. 3 *Energetic and cost aspects analysis for the two configurations one-stage CH_4 and one-stage CH_4 combined with alkaline pretreatment.*

	Configuration 1 (CH_4)	**Configuration 2** ($NaOH$-CH_4)		
Energy produced (Heat and Electricity from CHP)(kWh t^{-1} TS)	1399	1769		
Heat produced (kWh t^{-1} TS)	700	884		
Heat increase (kWh t^{-1} TS)		185		
Electricity produced (kWh t^{-1} TS)	490	619		
Electricity increase (kWh t^{-1} TS)		129		
Heat Energy requirement for pretreatment	**solid loading (kg TS m^{-3})**		**35**	**200**
Heat Energy requirement (kWh t^{-1} TS)			1034	210
Heat Energy requirement with 80 % of heat recovery (kWh t^{-1} TS)			207	42
Economic balances				
Electricity energy gain (€ t^{-1} TS) France	103	130		
Electricity energy gain (€ t^{-1} TS) Germany	122	155		
Electricity energy gain (€ t^{-1} TS) Italy	137	173		
NaOH cost (€ t^{-1} TS)		17		
Extra net gain (€ t^{-1} TS), France		10		
Extra net gain (€ t^{-1} TS), Germany		16		
Extra net gain (€ t^{-1} TS), Italy		19		

Then economic aspects of pretreatment implementation were considered comparing configuration 1 and 2 (Table VII.3). As it was commun to both configurations, heating of digester was not considered. In addition, we consider that methane production after alkaline pretreatment will provide enough heat for pretreatments requirements. Thus thermal pretreatment cost was considered as null.

As first assumption, three government incentive policies for biogas energy were considered in three European countries: France (0.21 € kWh^{-1}_{el}), Germany (0.25 € kWh^{-1}_{el}), and Italy (0.28 € kWh^{-1}_{el}). As second assumption, the European cost of the sodium hydroxide was used (412 € ton^{-1} (ICIS, 2010)).

The net gain was calculated for configuration 2, by considering the NaOH cost and the electric gain obtained by the extra electric production in configurations 2 compared to the untreated one (configuration 1).

Results are presented in Table VII.3 for three European countries. Net gains of 10 € t^{-1} TS, 16 and 19 € t^{-1} TS were respectively observed for France, Germany and Italy, showing that the net gain is mainly dependent on the call price which fluctuates from one country to another.

4. Conclusions

In this chapter, two configurations: one-stage CH_4 with alkaline pretreatment step and two-stage H_2/CH_4 were compared to anaerobic digestion alone (one-stage CH_4). Alkaline pretreatment improved the total energy (+26.5 %) for one-stage-CH_4 process. In contrast, two-stage H_2/CH_4 process did not improve total energy gain compared to one-stage CH_4 process. Thus energy and economic aspects have been studied only in one-stage CH_4 with or without alkaline pretreatment.

Considering the conversion of the biogas through a combined heat and power (CHP) with a thermal efficiency of 50 % and an electricity efficiency of 35 %, net heat production was deficient of 849 kWh t^{-1} TS at low solid loading of 35 kg TS m^{-3}. By considering the same solid loading and 80% of heat recovery from the pretreatment step, net heat production was almost null. On the contray, net heat production quite equivalent (-25 kWh t^{-1} TS) and positive (143 kWh t^{-1} TS) were observed at a higher solid loading of 200 kg TS m^{-3} without or with 80% of heat recovery. Indeed increase the solid loading of pretreatment permits to reduce the heat requirement expressed in kWh t^{-1} VS due to the step

pretreatment. However, these calculations have been realized on the assumption that performances are the same at solid loading of 200 kg TS m^{-3} than 35 kg TS m^{-3}.

Economic aspects were thus considered only by the sale of electricity according the call price of three European countries: France, Germany and Italy. A net gain varying form 11€ t^{-1} TS to 23€ t^{-1} TS was observed suggesting that the economic feasibility of combining alkaline pretreatment with one-stage CH_4 can be supported.

Nevertheless, these economic aspects give only estimation for the feasibility at industrial scale. Indeed, a more rigorous economic study should be realized taking into account the investment of infrastructure, and additional costs due to alkaline pretreatment.

Chapter VIII. General conclusions and outlook

Second generation biofuels, such as biohydrogen and methane, that are produced from lignocellulosic materials (ie. agricultural residues, grass, energy crops...), appear to be one of the most promising alternatives to fossil fuels with no "food versus fuel" dilemma. Contrary to first generation biofuels that use the edible part of plant, second generation are produced from lignocellulosic biomass, mainly agricultural residues or energy crops cultivated in soils that are unsuitable for food production. Biohydrogen and methane can be produced through fermentative microbial processes called dark fermentation and anaerobic digestion, respectively. The production of a "biohythane" gas, a mixture of hydrogen (5-20%) and methane (80-95%), through two-stage H_2/CH_4 biological process appears also very interesting. Indeed, recent studies showed that the addition of hydrogen to methane results in a more stable combustion and in a lower exhaust gases emissions level than that obtained by using methane alone (Sierens and Rosseel, 2000). However, the chemical and structural characteristics of lignocellulosic materials are limiting the microbial accessibility to carbohydrates for further conversion to biohydrogen or methane production.

The first objective of this study was to investigate the compositional and structural features that limit the lignocellulosic conversion into biohydrogen and methane. For this purpose, six compositional and structural characteristics, ie soluble carbohydrates (soluble carbohydrates coming from sucrose, starch or unilin), crystalline cellulose, amorphous holocelluloses (amorphous cellulose + hemicelluloses), lignin, protein and uronic acids were characterized from a panel of twenty different lignocellulosic materials. Then, biohydrogen and methane yields were experimentally determined and it was shown using Partial Least Square regressions that they correlated to several compositional and structural characetristics. First, hydrogen yields correlated positively with soluble carbohydrates only. In contrast, methane yields correlated negatively with the lignin content and in a lesser extent with the crystalline cellulose, but positively with the soluble carbohydrates, the amorphous holocelluloses and the protein content. Besides giving a quick tool to predict biohydrogen and methane yields from lignocellulosic substrates, these models were also valuable to give directions for the development of new pretreatments strategies of lignocellulosic residues for further enhancing both biohydrogen and methane production.

Sunflower stalks having a recalcitrant structure to biological attack (high lignin and low soluble carbohydrate contents) were chosen for further application of pretreatments (thermo-alkaline, enzymatic and combination of them). The objective was to investigate the compositional and structural changes of lignocellulosic components during such pretreatments. Among the thermo-chemical pretreatments, acid pretreatments ($FeCl_3$ and HCl) were found efficient in solubilizing hemicelluloses and uronic acids. In contrast, alkaline and oxidative pretreatments were more effective in dissolving lignin ($Ca(OH)_2$, H_2O_2 and NaOH). Enzymatic pretreatment (cocktail of cellulase, hemicellulases and β-glucosidase) was found efficient to increase the solubilization and conversion of holocelluloses to monomeric sugars. The enzymatic pretreatment was improved when combined alkaline with enzymatic pretreatment since the alkaline pretreatment increased the holocelluloses accessibility to enzymes and thus, their further conversion into soluble monomers sugars.

These results on the effect of pretreatments on lignocellulosic matrix coupled with the results obtained on structural and compositional characteristics that affects biohydrogen and methane production have permitted to develop new pretreatment strategies. For hydrogen production, an increase of the soluble carbohydrates content is required: acid and combined alkaline-enzymatic pretreatments which were found efficient in hydrolyzing hemicelluloses and part of the cellulose were considered for the improvement the hydrogen production. Similarly, several factors were found to influence the methane production of lignocellulosic substrates such as reducing lignin and increasing soluble carbohydrates contents, and in a lower extent reducing the crystalline cellulose content. Thus, all kinds of thermo-chemical pretreatments previously investigated were considered to enhance methane potential of the sunflower stalks.

For hydrogen potential, after dilute-acid pretreatment (170°C, 4% HCl), some byproducts (furfural, 5-HMF and phenolic compounds) were found in the hydrolysate and were responsible for the inhibition of the hydrogen fermentation. By adding gradual volumes of this hydrolysate in a control with glucose as sole carbon source (5g L^{-1}), a decrease of the hydrogen yield was observed. This decrease resulted in a metabolite shift from acetate/butyrate toward ethanol/lactate fermentation. Data from microbial community dynamic analysis demonstrated that new clusters of microorganisms, corresponding to non clostridial species, emerged and became predominant according to the increasing volume of hydrolysate added in the

control. On the contrary, combined alkaline-enzymatic pretreatment was shown efficient to enhance hydrogen production. Indeed, alkaline pretreatment before enzymatic hydrolysis increased the enzymatic accessibility to holocelluloses resulting in a higher release of soluble sugars which is the condition required to enhance hydrogen production. Among the thermo-chemical pretreatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$), alkaline pretreatment was found to be the best method to enhance methane potentials of sunflower stalks at a concentration of 4% NaOH (w/w), temperature of 55°C and time of 24 h. Moreover, this condition was found efficient on four different sunflower stalk samples with different but relatively close chemical compositions. Indeed, the methane potentials of these samples increased from 29 % to 44 %. Thus, alkaline pretreatment which has the main effect on delignification can be strongly recommended to enhance methane potentials from lignocellulosic residues.

In the last part of this work, the best pretreatment conditions for methane production (55°C, 24 h, 4% NaOH) defined previously in the batch assays were tested in anaerobic mesophilic continuous reactors to evaluate the feasibility at industrial scale. Normally, to be advantageous and economic, dark fermentation have to be coupled with another process able to convert the metabolites produced mainly acetate and butyrate. A promising process is the association of hydrogen with methane production in a two-stage process. Thus, coupling two-stage H_2 (batch) / CH_4 (continuous) was also investigated compared to one-stage CH_4 (continuous). In term of energy produced, no significant difference was observed between one-stage CH_4 and two stage H_2 / CH_4. In contrast, alkaline pretreatment was found efficient to enhance by 26.5% total energy produced compared to the one stage-CH_4 process. However, only 48 % of VS was removed after anaerobic digestion of alkaline pretreated sunflower stalks suggesting that a part of the organic matter was still not accessible and degradable. In addition, economic assessment showed that coupling alkaline pretreatment with anaerobic digestion process compared to anaerobic digestion alone can lead to a net gain varying from 11 € t^{-1} TS (France) to 20 € t^{-1} TS (Italy). Nevertheless, these economic aspects give only estimation for the feasibility at industrial scale. Indeed, a more rigorous economic study should be realized taking into account the investments in infrastructures, and the additional costs due to the pretreatment step or the two-stage H_2 / CH_4 technologies.

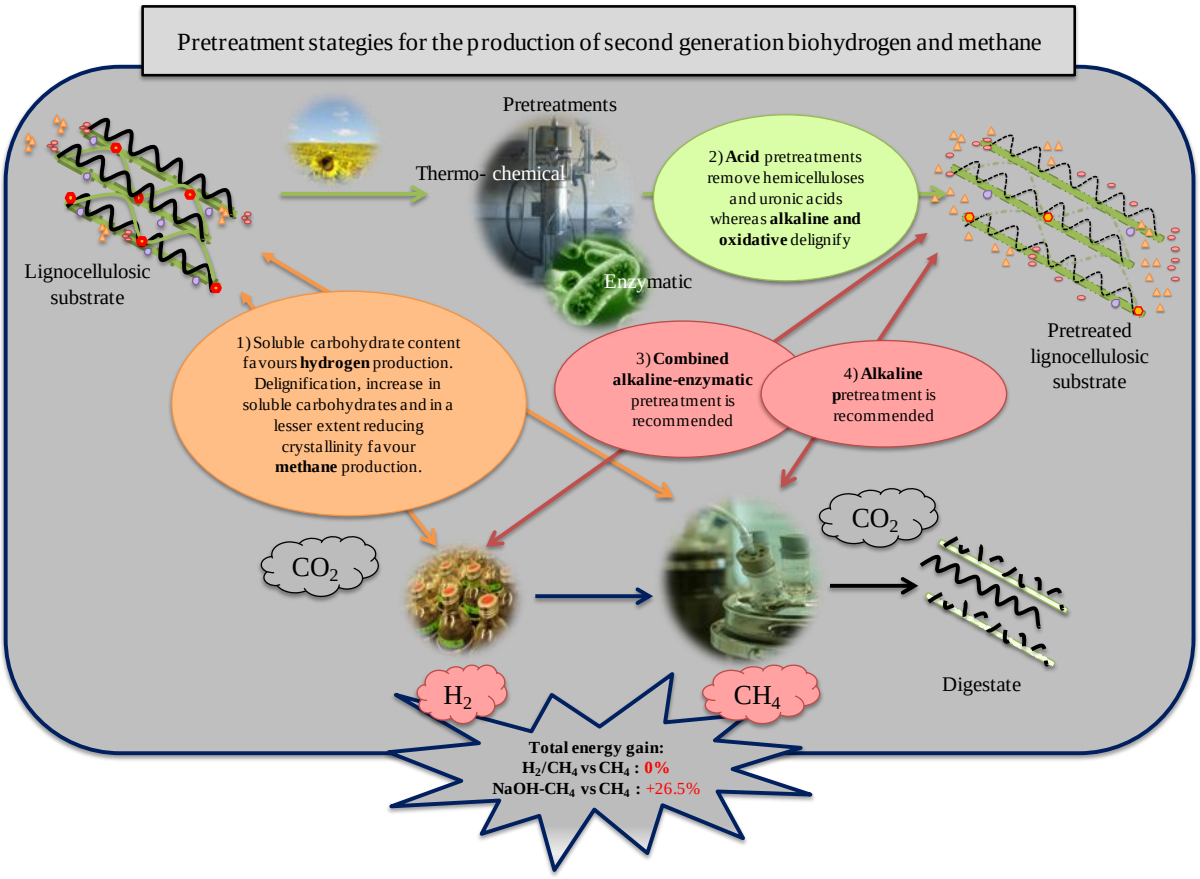

Figure VIII. 1 *Overall scheme of the main conclusions of this thesis.*

Through an interdisciplinary approach, this work has contributed to improve the understanding of compositional and structural features of lignocellulosic materials that limit their further conversion into biohydrogen or methane (Figure VIII.1). Based on these results, pretreatments strategies, have been defined to improve both hydrogen and methane production. However, several bottlenecks have to be resolved to understand and avoid that a part of the biodegradable still remains in outlet of the anaerobic continuous reactors. Predictive models defined for biohydrogen and methane production can be improved. Even though pretreatment strategies have been defined both for hydrogen and methane some other pretreatments deserve to be investigated. Moreover, future work on the improvement of enzymatic pretreatments and the optimization of thermo-chemical pretreatment parameters (solid loading, time, temperature, concentration of chemical reagent) has to be considered to make the process more economically-viable. In this sense, several perspectives and future works can be suggested:

1) To improve the understanding in the compositional and structural features that affect hydrogen or methane production, the previous PLS models can still be considered as restrictive. These models could therefore be improved by considering more chemical and structural characteristics, such as the contents of acetyl groups and soluble proteins, the surface area and pore volume. Indeed some of these parameters have been found to act significantly on enzymatic hydrolysis of lignocellulosic substrates (Chang and Holtzapple, 2000). Moreover, innovative technique as fluororescence 3D could be considered to characterize more accurately the soluble fraction such as protein or phenolic compounds released during pretreatments.

2) Regarding the compositional and structural characteristics that limit lignocellulosic conversion into biohydrogen and methane, pretreatment strategies have been defined but other pretreatments deserve to be investigated. As for methane production, crystalline cellulose was found to have a negative impact, pretreatments which are normally known to reduce cellulose crystallinity as milling or ionic liquid could be investigated (Samayan et Schall, 2010; Gharpuray et al., 1983). Considering hydrogen production, acid pretreatment using inorganic salts such as $FeCl_3$ can be also an interesting alternative method as it was shown in this work that such pretreatment are efficient in hydrolyzing hemicelluloses into soluble carbohydrates.

3) Several bottlenecks remain on the application of enzymatic pretreatment such as aseptic conditions and high cost of enzymes. First, aseptic conditions required for enzymatic pretreatment constitute a strong barrier for future development at industrial scale. To avoid a sterilization step, the carbon dioxide produced in the biogas can be reused to generate oxygen free environment and avoid proliferation of aerobic microorganisms that consume free sugars released during enzymatic pretreatment. Another solution is the simultaneous addition of enzyme mixture on the fermentative reactor for concomitant release and degradation of the sugars as previously suggested by Romano et al. (2009) and Quémeneur et al. (2012). Then, to free oneself of the high cost of industrial enzymes, the use of biological pretreatment such as

fungi can be a promising alternative for both hydrogen and methane production. Indeed, some fungi are able to generate cellulosic, hemicellulosic and lignolytic enzymes (Dashban et al., 2009).

4) Maximizing hydrogen or methane is an important aspect to make the process economically-viable. Thus, optimization of parameters (solid loading, time, temperature, concentration of chemical reagent) has to be considered. First, it has been highlighted that the heat requirement of thermal pretreatment is dependent on the solid loading used: the higher is solid loading, the less is heat requirement. Thus, it could be interesting to evaluate the impact of high solid loading (≥ 15 % (w/w) on pretreatments performances in particular compositional and stuctural changes, and biohydrogen or methane production.

   Then, optimization of parameters (time, temperature, concentration of chemical reagent) to maximize hydrogen or methane potential is necessary to make the process economic. However, optimization of thermo-chemical parameters is a time consuming work that can be simplify using Experimental design. Nonetheless, optimization of thermo-chemical parameters depends on the substrates (ie compositional and structural features) and can not be extrapolated to one to another substrate. And yet, industrial digesters are fed with a large range of lignocellulosic substrates (crop rotations or ensiling) during all the year with different structural and compositional features. Thus it could be interesting to optimize pretreatments parameters directly as regards to a large range of compositional and structural features present in various lignocellulosic substrates. Further models could be build between the pretreatments parameters and the compositional and structural features. Consequently, according the compositional and structural features of the investigated lignocellulosic substrates, it could be possible to choose the appropriate parameters conditions to maximize both hydrogen and methane production.

5) Several perspectives can be considered also for continuous reactors. It should be interesting to extent batch hydrogen production to continous mode in two-stage H_2/CH_4 and to test the two-stage H_2/CH_4 configuration with substrates adapted to hydrogen production with high soluble carbohydrates content such as Jerusalem artichoke or sorghum. Furthermore, pretreatment step prior H_2 stage or between H_2 and CH_4 stage can be investigated. Prior H_2 stage, a pretreatment that acts on the solubilization of holocelluloses should be considered to enhance hydrogen production and between H_2 and CH_4 stage pretreatment that acts more on the delignification have to be favoured.

6) Finally, in a perspective of developing the concept of biorefinery that provides a large range of bio-based products, supplementation of dark fermentation cultures by byproducts like furans and phenolic compounds can be used as a strategy to turn toward other value added products such as ethanol and lactate. Moreover, it should be interesting to consider the valorization of the digestate. One solution can be the use of the digestate directly as agricultural fertilizer. However, this digestate which is rich in lignin can be used for the production of value added products such as phenolic compounds. It could be interesting to characterize the quality of lignin at the end of reactors according the pretreatment performed and the fermentative processes used (one-stage CH_4 or two-stage H_2/CH_4).

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Abstract

In the future, various forms of renewable energy, such as second generation biofuels from lignocellulosic residues, will be required to replace fossil fuels. Among these, biohydrogen and methane produced through fermentative processes appear as interesting candidates. However, biohydrogen and/or methane production of lignocellulosic residues is often limited by the recalcitrant structure and a pretreatment step prior to fermentative processes is often required. Up to date, information on lignocellulosic characteristics limiting both hydrogen and methane production is rather limited. Therefore, this work aims to investigate the effect of compositional and structural features of lignocellulosic residues on biohydrogen and methane performances for further developing appropriate pretreatments strategies. Firstly, a panel of twenty lignocellulosic residues was used to correlate both hydrogen and methane potentials with the compositional and structural characteristics. The results showed that hydrogen potential positively correlated with soluble carbohydrates only. Secondly, methane potential correlated negatively with lignin content and, in a lesser extent, with crystalline cellulose, but positively with the soluble carbohydrates, amorphous holocelluloses and protein contents. Pretreatments strategies were further developed to enhance both hydrogen and methane production of sunflower stalks. Dilute-acid and combined alkaline-enzymatic pretreatments, which were found efficient in solubilizing holocelluloses into soluble carbohydrates, were applied prior to biohydrogen potential tests. By combined alkaline-enzymatic pretreatment, hydrogen potential was fifteen times more than that of untreated samples. On the contrary, hydrogen production was inhibited after dilute-acid pretreatments due to the release of byproducts (furfural, 5-HMF and phenolic compounds) that led to microbial communities shift toward no hydrogen producing bacteria. Similarly, methane production, five thermo-chemical pretreatments (NaOH, H_2O_2, $Ca(OH)_2$, HCl and $FeCl_3$) found efficient in delignification or solubilization of holocelluloses, were considered. Among these pretreatments, the best conditions were 55°C with 4% NaOH for 24 h and led to an increase of 29-44 % in methane potential of sunflower stalks. This pretreatment condition was validated in one stage anaerobic mesophilic continuous digester for methane production and was found efficient to enhance from 26.5% the total energy produced compared to one stage-CH_4 alone. Two-stage H_2 (batch) / CH_4 (continuous) process was also investigated. Nevertheless, in term of energy produced, no significant differences were observed between one-stage CH_4 and two-stage H_2/CH_4.

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