Fungal pretreatments for lignocellulosic biomass anaerobic digestion

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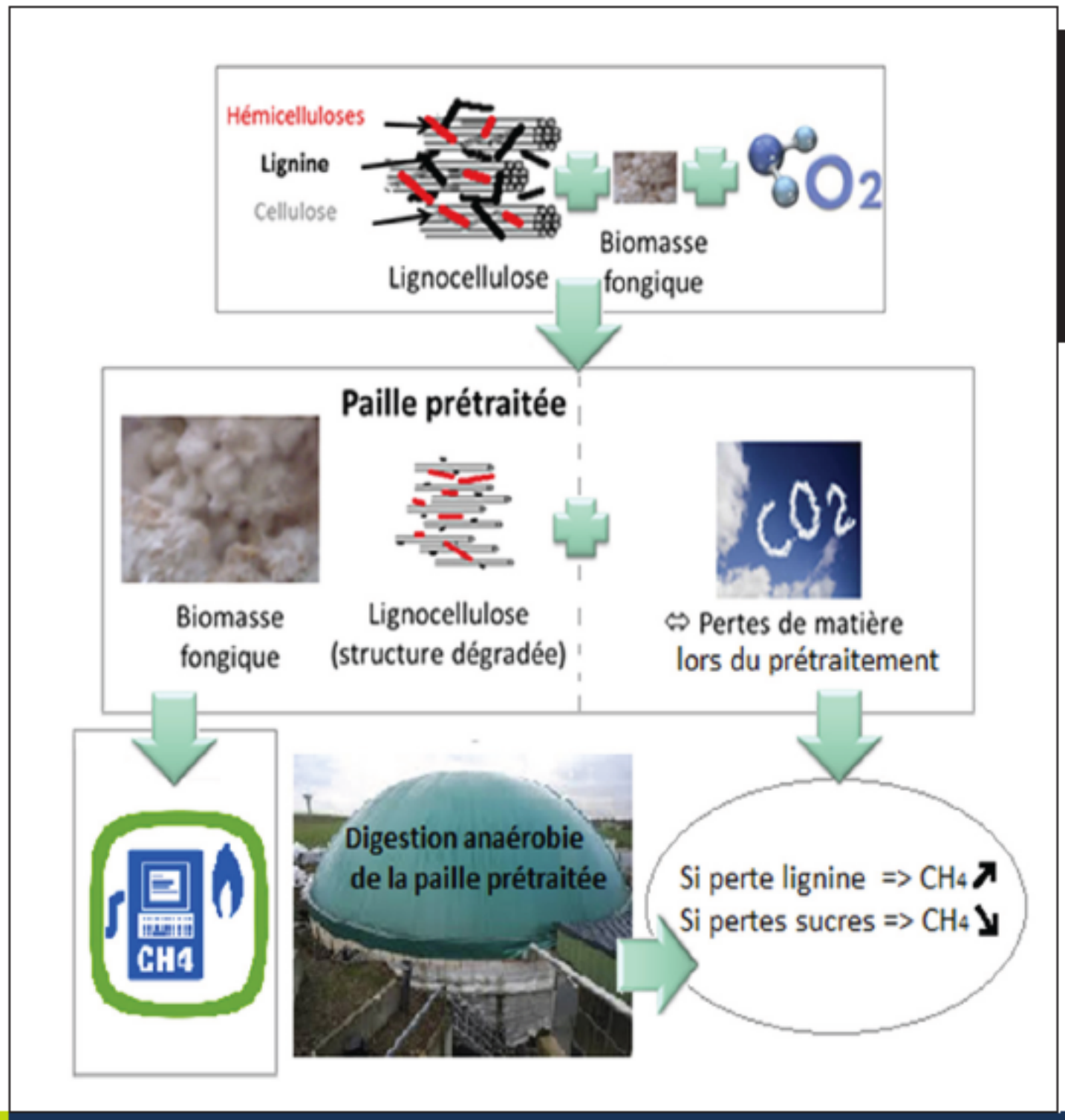

PhD thesis 2015

PRÉTRAITEMENTS FONGIQUES POUR LA MÉTHANISATION DE LA BIOMASSE LIGNOCELLULOSIQUE

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2015

FUNGAL PRETREATMENTS FOR LIGNOCELLULOSIC BIOMASS ANAEROBIC DIGESTION

INRA SCIENCE & IMPACT

Membre fondateur de agreenium

Collège Doctoral du Languedoc-Roussillon

THÈSE

Pour obtenir le grade de

Docteur

Délivré par **Université de Montpellier**

Préparée au sein de l'école doctorale **GAIA**
Et de l'unité de recherche **Laboratoire de Biotechnologie de l'Environnement** (INRA, UR0050 LBE)

Spécialité : **APAB - Agroressources, Procédés, Aliments, Bioproduits**

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Prétraitements fongiques pour la méthanisation de la biomasse lignocellulosique

Fungal pretreatments for lignocellulosic biomass anaerobic digestion

Soutenue le 17/12/2015 devant le jury composé de

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Université de Montpellier

A ma grand-mère,

Idalina Amélia FERREIRA LOPES

Abstract

Anaerobic digestion of lignocellulosic biomass is one of the most efficient ways to produce renewable energy. However, lignin contained in this biomass is difficult to hydrolyze. This limitation can be overcome by pretreatments. Among them, low-cost white-rot fungi pretreatments seem attractive but were scarcely applied for anaerobic digestion. The current study investigates white-rot fungi pretreatments of wheat straw to improve its methane production. Firstly, a selection step has revealed the efficiency of *Polyporus brumalis* BRFM 985 since 43% more methane per gram of pretreated volatile solids were obtained compared to the control straw. Taking into account the dry weight loss occurring during the pretreatment, it still corresponded to 21% more methane per gram of initial total solids. Moreover, glucose addition during the pretreatment was shown to limit delignification and thus methane production from the substrate. Secondly, pretreated samples were obtained in an experiment device aiming to optimize the pretreatment with *P. brumalis* BRFM 985; tested pretreatments parameters were: culture duration, temperature, initial substrate moisture content and metals addition. Response surfaces of methane production from those samples were built. Optimum methane production was not reached in the experimental domain but the positive impact of metals addition was demonstrated, so as the importance to choose adequate culture duration. Then, the use of pyrolysis-GC-MS technic to access pretreatment efficiency was studied. Estimation of fungal biomass amount on wheat straw with this method appeared possible. Polysaccharides/lignin ratio determined with py-GC-MS allowed to classify some pretreated samples according to their anaerobic degradability. Solid State Anaerobic Digestion (SSAD) of wheat straw pretreated in pilot-reactor was carried out in batch with leachate recycle. During SSAD start-up phase, too high Substrate/Inoculum (S/I) ratio leads to Volatile Fatty Acid (VFA) accumulation and sometimes to reactor failure but with high S/I more substrate can be treated and methane production per reactor volume increases. With wheat straw, S/I between 2 and 3 (Volatile Solid basis) allow a successful start-up in SSAD. Whereas Total VFA/alkalinity ratio under 0.6 corresponds to stable wet anaerobic digestion; this limit seems not well adapted to SSAD. It was observed that SSAD reactors were able to recover from acidification phase when Total VFA/alkalinity was lower than 2 and with VFA concentrations inferior to 10 g/L in leachate. Despite the improvement of biodegradability and the facilitation of start-up phase, non-optimized fungal pretreatment did not improve methane production after taking into account mass losses occurring during the pretreatment.

Keywords: *White-Rot Fungi (WRF); wheat straw; Solid State Anaerobic Digestion (SSAD); Solid-State-fermentation (SSF); culture conditions; delignification; py-GC-MS; methane;*

Résumé

La méthanisation de la biomasse lignocellulosique est un des moyens les plus efficients pour la production d'énergie renouvelable. Cependant, la lignine présente dans cette biomasse est difficile à hydrolyser. Cette limite peut être surmontée grâce aux prétraitements. Parmi eux, les prétraitements peu couteux par pourritures blanches sont attrayants mais ils ont été peu appliqués pour la digestion anaérobie. La présente étude explore les prétraitements par pourritures blanches de la paille de blé afin d'en améliorer sa méthanisation. Tout d'abord, une étape de sélection a révélé l'efficacité de la souche *Polyporus brumalis* BRFM 985 puisque 43% de méthane supplémentaire ont été obtenus par gramme de matières volatiles par comparaison avec la paille témoin. En prenant en compte les pertes de matières occasionnées par le prétraitement, cela correspondait à 21 % d'amélioration par gramme de matière sèche initiale. De plus, il fut montré que l'addition de glucose durant le prétraitement limitait la délignification et donc la production de méthane du substrat. Puis, des échantillons prétraités ont été obtenus lors d'un plan d'expérience visant à optimiser le prétraitement par *P. brumalis* BRFM 985 ; les paramètres du prétraitement testés étaient : la durée et la température de culture, l'humidité initiale du substrat et l'addition de métaux. Les surfaces de réponse de la production de méthane à partir de ces échantillons ont été construites. La production optimale de méthane n'a pas été atteinte dans le domaine expérimental testé mais l'impact positif de l'addition de métaux a été démontré, ainsi que l'importance de choisir une durée de culture adaptée. Ensuite, l'usage de la technique de la pyrolyse-GC-MS pour évaluer l'efficacité du prétraitement a été étudié. Une estimation de la quantité de biomasse fongique avec cette méthode apparaît possible. Le ratio polysaccharides/lignine déterminé par py-GC-MS a permis de classer des échantillons prétraités selon leur biodégradabilité anaérobie. La digestion anaérobie en voie sèche (DAVS) de paille de blé prétraitée en réacteur pilote a été menée en batch avec recirculation des lixiviats. Durant le démarrage de la DAVS, un trop fort S/I mène à une accumulation d'acides gras volatils (AGV) et parfois à la défaillance de la DAVS. Néanmoins, de forts S/I permettent de traiter plus de substrat et augmentent la production de méthane par volume de réacteur. Avec la paille de blé, des S/I entre 2 et 3 (en matières volatiles) permettent un bon démarrage de la DAVS. Alors qu'un ratio AGV totaux/alcalinité inférieur à 0,6 correspond à des réacteurs stables en digestion anaérobie voie liquide ; cette limite semble mal adaptée à la DAVS. Il a été observé que la DAVS pouvait récupérer d'une phase d'acidification tant que le ratio AGV totaux/alcalinité était inférieur à 2 et que la concentration en AGV était inférieure à 10 g/L dans les lixiviats. Malgré une amélioration de la biodégradabilité et une phase de démarrage facilitée, le prétraitement fongique non optimisé n'a pas permis d'améliorer la production de méthane après prise en compte des pertes de matière occasionnées par le prétraitement.

Mots-clés: *pourritures blanches ; paille de blé ; digestion anaérobie en voie sèche ; Fermentation en Milieu Solide (FMS) ; conditions de culture ; délignification ; py-GC-MS ; méthane ;*

Remerciements

Je remercie tous les membres du projet Stockactif et plus particulièrement les personnes qui ont eu la gentillesse de me fournir quelques informations utiles : Francis DUCHIRON (UMR FARE), Gérard LAINE (Vivescia), Pauline RAPPINAT et Anaïs GUILLAUME (Soufflet). Les expérimentations et échanges avec Mathieu MULLER (Envolure) et Abdellatif BARAKAT (UMR IATE) se sont également révélées enrichissants. Merci à l'INRA et à l'ANR pour m'avoir financée et avoir cru en ce projet. Merci à Marie-France DIGNAC (UMR EcoSYS) pour sa participation aux travaux de pyrolyse et pour m'avoir formée à cette technique. La collaboration avec l'UMR BBF et notamment avec Simeng ZHOU a aussi fortement contribué à étayer mes connaissances sur les champignons. Je remercie Michelle SERGENT pour l'élaboration du plan d'expérience. J'ai également particulièrement apprécié les encouragements et conseils d'Isabelle GIMBERT. Grâce à elle, j'ai pu accéder à des résultats expérimentaux indispensables à cette thèse (qPCR et deep-well avec différentes quantités de glucose). Elle s'est également impliquée dans mon comité de thèse, la relecture de ma revue bibliographique ou encore de ma thèse en tant que membre du jury.

J'en viens donc à remercier les autres membres de mon comité de thèse pour avoir pris le temps de s'intéresser à mon travail : Jean TAYEB (UMR IATE), Richard AURIA (IRD) et Sylvaine CRAPART (ARD). Finalement, j'ai aussi une pensée pour les autres membres de mon jury de thèse Christelle WISNIEWSKI (Université de Montpellier), Rémy BAYARD (INSA de Lyon), Thierry RIBEIRO (Institut Polytechnique LaSalle Beauvais) et Maritxu GUIRESSE (INP ENSAT). Je les remercie pour leur temps, conseils et encouragements. Je garderai un bon souvenir d'eux et aussi bizarre que cela puisse paraître de ma soutenance.

Bien entendu, je te remercie de tout cœur Hélène CARRERE (UR LBE), ma directrice de thèse, pour ton investissement important dans mon travail, tes connaissances, ta pertinence, ta porte toujours ouverte, ton calme, ton honnêteté, ta confiance… Peut-être m'auras-tu transmis un peu de ta patience ? Je suis vraiment heureuse et reconnaissante d'avoir passé ces trois années sous ta tutelle. Merci aussi pour la Grèce où nous et Denis LOISEL avons passé de bons moments, ce fut agréable d'échanger avec vous. Merci pour le Chili, je suis sortie de l'Europe pour la première fois. La main de Jean-Phillipe STEYER, que je tenais dans l'avion s'en rappellera. Jean-Phi, tu as toujours cru en moi et m'a orientée dans mes moments difficiles.

Eric LATRILLE, cela serait long de tout dire entre les sorties canoë à renfort de bébés goélands et de taboulé ainsi que les ateliers groupe de rock agrémentés d'interminables discussions sur comment travailler proprement dans un milieu moyennement propre, etc. J'ai particulièrement apprécié ta gaité et ta bienveillance notamment envers les « jeunes » ; ton esprit toujours en ébullition et tes grandes compétences en statistiques m'ont également été précieuses. Renaud ESCUDIE, je retiendrai ta bonne humeur et surtout ta patience pour m'avoir toujours expliqué deux fois ce que tu me disais (j'étais pas très en forme en fin de thèse ☺).

Thierry CONTE, ce fut un plaisir de te cotoyer en début de thèse. Merci pour ton altruisme et la transmission d'une partie de tes connaissances dans la gestion de l'HPLC. Cela me fait penser que je pourrai en dire autant de Nadine DELGENÈS. J'ai aussi une pensée pour mes stagiaires, Marie-Aude SOULA et Rita SCHUBERT, dont j'ai su apprécier la motivation et le sérieux.

Je souhaiterais sincèrement remercier tous les membres de mon laboratoire pour leur aide, empathie et soutien. Je ne pourrai probablement pas tous vous citer mais chacun de vos petits gestes m'ont toujours été droit au cœur qu'il s'agisse du chocolat noir de Frédéric HABOUZIT, de la réparation de mon pare-choc par Guillaume GUIZARD ou de l'aide de Marjolaine HAMELIN pour les reliures de mes thèses, etc. Merci, Véronique MAUGENET pour m'avoir trouvé des multitudes de publis introuvables même si elles dataient de 1960. Merci, Philippe SOUSBIE, expert en bricolage de réacteurs et plus encore… Merci Nori HENNAOUI-ROSAY pour le fond et la forme. Merci aux administratrices Sylvie FARINE, Nadine LE THINH, Alexandra MESEGUER et Annie VIDAL pour leur gentillesse.

J'ai durant ma thèse fait appel au moins une fois à chacun des permanents du laboratoire (désolée de ne tous vous citer), j'ai toujours reçu de bons conseils scientifiques et un bon accueil de la part de tous. Vous m'avez aussi tous beaucoup soutenue sur la difficile fin de ma thèse et avez contribué au succès de ma journée de soutenance. Je vous souhaite le meilleur, j'espère vous avoir rendu un peu de ce que vous m'avez donné et si ce n'est pas le cas, sachez que je compte bien m'améliorer.

Enfin, merci à l'échoppe pour son soutien moral et merci aux coupaings qui ont pu y venir avec moi. Une belle équipe qui a mis un peu de magie dans ces trois années : ceux qui sont partis, Mélanie PIERRA, Thibaut SAUR et ceux qui sont arrivés, Diane PLOUCHART, Wendy LAPERRIÈRE… Lister vos qualités et tout ce qu'on a pu faire risquerait d'être plus long que ma thèse elle-même… Alice DANEL, mon petit viking et ses brownckis (un mélange de cookies et de brownies, dooble fat, oui c'est possible), comment aurais-je pu faire sans ta senseo pour me tenir concentrée 10 heures par jour ? Marie-Lou HILLION, je ne pensais pas qu'un jour je pourrais aller courir sans y être contrainte et pourtant c'est maintenant une habitude grâce à toi. Je repense aussi aux balades en roller accompagnées par Antonella MARONE, sa sérenité et son humour. Angel ROBLES, mi osito, sería indecente de decir màs ;) Je compte bien passer te voir à Valencia un jour, peut-être accompagnée par le mignon Gabriel CAPSON et dark pipine. Julie JIMENEZ, mon agence conseil, parfois inadaptée mais toujours drôle et empathique. Florian PAILLET, mon premier fan à qui je dédierai mon unique futur single. Cyrille CHARNIER, Sissi, je retiendrai entre autres ton sens de l'humour déplacé et tes questions indiscrètes… Roman MOSCOVIZ, merci pour ta patience et ton intelligence. Quentin AEMIG, mon pti Q d'amur, après avoir partagé notre goûter (^^), avoir dormi ensemble et t'avoir déguisé en fille, j'ai fini par assister à ton mariage ! Lucile CHATELLARD, Lulu, ma co-bureau (des plaintes), t'en as parcouru du chemin depuis ton stage ! Tout ira bien, promis. J'espère être là pour ta soutenance, merci de t'être si bien occupée de moi. Elisabeth CAZIER et Violette TURON, mes compagnonnes de galère… Zaza, même si ce fut parfois douloureux, j'admire ta générosité infinie. Vio, les soirées sans toi c'est moins rigolo. J'espère qu'on fera encore pleins de voyages en namoureuses pour manger plein de gras ☺. Je suis contente de t'avoir eue à mes côtés durant ces trois ans et après mes rupturesss, je te retourne tes mots « sans toi c'aurais pas été pareil, » et j'ajoute « beaucoup moins bien ».

Plein d'agréables moments avec vous, plein de belles personnes, vous me manquerez même si j'espère bien vous garder un peu dans ma vie !

Finalement, merci à mes parents, André et Isabel ROUCHES, notamment pour m'avoir transmis la valeur de l'honnêteté et du travail et pour avoir toujours fait de leur mieux pour nous élever mon frère Sébastien et moi. Merci également à Sébastou pour son soutien inconditionnel et sa gentillesse. Cette thèse c'est un peu pour vous.

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3D: 3 dimensions
2w: two weeks (pretreatment duration)
A: arabinose
AAO: aryl-alcohol oxidase.
ABTS: Acide 2,2'-azino-bis (3ethylBenzoThiazoline-6-Sulphonique)
AD: Anaerobic Digestion
ADD: aryl-alcohol dehydrogenases.
AFEX: Ammonia Fiber EXplosion
Am: Amorphous holocelluloses
ANOVA: ANalysis Of Variance
ANR: Agence Nationale de Recherche
APO: Aromatic PerOxygenase
BBF: Biodiversité et Biotechnologies Fongiques
BMP: Biochemical Methane Potential
BRF: Brown-Rot Fungi
BRFM: Banque de Ressources Fongiques de Marseille, Bank of Fungal Ressources of Marseille
Cel: Cellulose
CIRM-CF: Centre International de Ressources Microbiennes-Champignons Filamenteux
cMWS: fungal colonized Miscanthus and Wheat Straw, and the corresponding batch
COD: Chemical Oxygen Demand
Cri: Crystalline cellulose
CrI: Crystallinity Index
Cs: *Cyathus stercoreus*
Cster: *Cyathus Stercoreus* AW 03-72
Csub: *Ceriporiopsis subvermispora*
d: days
DAVS: Digestion Anaérobie Voie Sèche
DCPIP: 2,6-DiChloroPhenol-IndoPhenol)
DFRC: Derivatization Followed by Reductive Cleavage
DHP: DeHydrogenation Polymer
DML: Dry matter losses
DMSO: DiMethylSulfOxide.
DNA: DeoxyriboNucleic Acid
DNS: DiNitroSalicylic acid method
DOF: Degree Of Freedom
DP: degree of polymerization
Ds: *Dichomitus squalens*
DTG: Differential ThermoGravimetry
DyP: DYe-decolorizing Peroxidase
ECOSYS: Ecologie fonctionnelle et écotoxicologie des agroécosystèmes
EDA: EthyleneDiAmine.
EDTA: EthyleneDiamineTetraAcetic acid
Ev. ElectronVolt
FA: ferulic acid
FARE: Fractionnement des Agroressources et Environnement
FTIR: Fourier-Transform InfraRed
FWS: Fungal pretreated Wheat Straw and the corresponding batch
G: guaiacyl-lignin units
GC: Gas Chromatography
Gf: Grifola frondosa
GLOX: glyoxal oxidase.
H2O2-GO: H2O2-generating oxidases.
Hc: Hericium clathroides
HCA: Hierarchical Cluster Analysis
Hemi: Hemicelluloses
HPLC: High Performance Liquid Chromatography
HSD: Honest Significant Difference
i. d.: internal diameter
i: initial
IATE: Ingénierie des Agro-polymères et Technologies Émergentes
Id: lnonotus dryophilus
INRA: Institut National de Recherches Agronomiques
Ir: Inonotus rheades
ITS (Internal Transcribed Spacer)
IVDMD: In vitro Dry Matter Digestibility
KL: Klason lignin
kPET (Petroleum Equivalent Ton)
LAC: laccases
LB: Lignocellulosic Biomass
LBE: Laboratoire de Biotechnologie de l'Environnement
LCC: Lignin-carbohydrates complex
LDS: Lignin Decomposing Selectivity
LIG: Lignin
Lig: Lignin
PS1/LIG: PS (Area at 1375 cm^{-1}) /Lig (1512 cm^{-1})
PS2/LIG: PS (1158 cm^{-1}) /Lig (1512 cm^{-1})
LiP: Lignin Peroxidase
LMS: Laccase-mediator system.
LOI: Lateral Order Index
M: Molar mass
MC: Moisture Content
MH: Mzsse Humide
Mip: Manganese independent peroxidase
MnP: Manganese Peroxidase
MS: Mass Spectrophotometry ou Masse Sèche *selon contexte*
MWS: Miscanthus pellets and Wheat Straw, and the corresponding batch
N. A.: Not Available
n: number of samples taken into account
NBO: NitroBenzene Oxidation
NIC: Non-inoculated SSF Control

NL: NormoLiters (Liters in Standard conditions for temperature and pressure)
NREL: National Renewable Energy Laboratory
OFMSW: Organic Fraction Municipal Solid Waste
OM: Organic Matter
PC: *Phanerochaete chrysosporium*
pCA: p-coumaric acid
PCA: Principal Component Analysis
Pci: *Pycnoporus cinnabarinus*
Pco: *Pleurotus cornucopiae*
PCS: Peptone Cellulose Solution
Pl : *Pleurotus liguatilis*
Pro: Protein
PS/LIG: polysaccharides (holocelluloses)/lignin
PS: Polysaccharides
Py-GC-MS: Pyrolysis-Gas Chromatography-Mass Spectrophotometry
Pyr: Pyrolysis
q. s.: quantum satis
qPCR: Quantitative Polymerase Chain Reaction
QR: quinone reductases
RI: Refractive Index
rpm: Revolutions Per Minute
RS: Response surface
RSM: Response surface methodology
RT: Retention Time
S. D.: Standard-Deviation
S/G: synapyl/ guaiacyl-lignin units
S/I: Substrate/Inoculum
S: synapyl-lignin units
SEBAC: Sequential Batch Anaerobic Composting
SGE: SolGelWax
SolSu: Soluble Sugars
Sr: *Stropharia rugosoannulata*
SSAD: Solid-State Anaerobic Digestion
SSF: Solid-State Fermentation
TC: Total Carbon
Temp: Temperature
Th: *Trametes hirsuta* AW 03-72
TKN: Total Kjeldahl Nitrogen
TS: Total Solids
TVFA: Sum of Volatile Fatty Acids expressed in acid acetic equivalents
Ua: Uronic acids
UMR: Unité Mixte de Recherche
UR: Unité de Recherche
UWS: Untreated Wheat Straw
VFA: Volatile Fatty Acids
vol: volume
VP: Versatile Peroxydase
VS: Volatile Solids
w: weight
WO: Wet Oxydation
WRF: White-Rot Fungi
wt: weight total
WW/TS: Wet Weight/Total Solid
X: xylose
XRD: X-ray diffractometry

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Patent

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Résumé étendu

Le réchauffement climatique, l'épuisement des ressources pétrolières, la compétition entre les cultures alimentaires et énergétiques et la diminution de la disponibilité des terres arables, sont autant de challenges mondiaux qui doivent être surmontés à l'heure actuelle. L'utilisation de substrats non-alimentaires, tels que la lignocellulose, devient donc une nécessité. De plus, la bioraffinerie n'est plus cantonnée à la production d'énergie puisqu'elle doit aussi fournir des réactifs chimiques ou encore des matériaux divers.

Le prétraitement de la lignocellulose est une étape clé afin de pouvoir combiner les divers usages. En effet, les végétaux sont constitués de trois polymères majoritaires : cellulose, hémicelluloses et lignine. Chacun de ces polymères peut couvrir un besoin. Le prétraitement permet d'isoler les fractions d'intérêt ou à minima de fragmenter les polymères qui limiteraient la production du produit recherché. Par exemple, les polysaccharides sont un substrat pour la méthanisation. Ils forment dans la paroi des végétaux la cellulose et les hémicelluloses. Cependant, la lignine (polyphénols) qui encercle ces polysaccharides est difficilement dégradable. Rompre la lignine va favoriser l'accès aux sucres et donc augmenter la production de méthane. Ainsi, le but principal du prétraitement pour la méthanisation va être la délignification de la matière. Chandler et *al.* (1980) ont montré qu'un pourcent de lignine entrainait une baisse de la digestion de la matière organique de l'ordre de 3% (Lesteur et *al.*, 2010).

Cependant, l'application industrielle des prétraitements est peu développée à l'heure actuelle. Elle est notamment limitée par des coûts de prétraitement excessifs. Les prétraitements sont généralement évalués par rapport à leur efficacité alors qu'en fait il est plutôt nécessaire d'avoir une balance économique positive ; c'est-à-dire que l'efficacité mesurable économiquement doit être supérieure aux coûts additionnels engendrés par le prétraitement. Néanmoins, si la production supplémentaire est en général facilement mesurable au laboratoire, l'estimation des coûts nécessite souvent des études à grandes échelles qui sont coûteuses et complexes à mettre en œuvre. Concernant la lignocellulose, l'explosion à la vapeur et les réactifs alcalins apparaissent comme les prétraitements les plus faisables à l'échelle industrielle (Hendriks and Zeeman, 2009). La durée requise pour qu'ils permettent un prétraitement efficace n'est que de quelques heures, voire quelques minutes. Pourtant, le contexte d'application doit également être pris en compte. En effet, l'explosion à la vapeur qui nécessite l'utilisation de hautes pressions est encadrée par des règles de sécurités strictes ce qui peut être plus problématique sur une exploitation agricole individuelle que sur un site industriel déjà soumis à des règlementations. Finalement, les impacts environnementaux potentiels doivent également être envisagés pour le choix d'un prétraitement. Par exemple, l'utilisation de produits chimiques nécessite parfois des étapes de récupération de ces produits qui sont coûteuses. Dans ce contexte, les prétraitements fongiques qui nécessitent une faible consommation d'énergie et de produits chimiques, et qui présentent donc un intérêt économique et environnemental, apparaissent intéressants par rapport aux autres types de prétraitements. Cependant, la durée nécessaire pour obtenir un prétraitement efficace est de plusieurs semaines. D'autres

part, certains substrats lignocellulosiques sont stockés durant une longue période d'où l'idée de mettre à profit cette période de stockage pour effectuer le prétraitement. Cette idée est à l'origine du projet ANR STOCKACTIF (2012-2016).

Actuellement, une période de stockage (par exemple, pour des pailles) est généralement suivie d'un prétraitement, puis de la valorisation de la matière. Avec le projet Stockactif un prétraitement fongique est réalisé durant le stockage. Les enjeux du projet sont de préserver un maximum de potentiel d'utilisation de la biomasse, d'identifier des conditions de stockage reproductibles et de favoriser la faisabilité industrielle. Ainsi, le projet s'intéresse à trois formes de valorisation de la biomasse : bioéthanol, biogaz et phénols. La valorisation en biogaz a été étudiée au Laboratoire de Biotechnologie de l'Environnement dans le cadre de cette thèse.

Le biogaz, constitué majoritairement de méthane et dioxyde de carbone est produit lors de la digestion anaérobie de la matière organique par des microorganismes. Alors que le méthane est convertible en énergie, le résidu solide, appelé digestat, est la plupart du temps valorisable sous forme d'engrais organique. La paille de blé, substrat modèle largement disponible et qui possède une composition représentative des biomasses lignocellulosiques (Vassilev et *al.*, 2012), a plus particulièrement été étudiée. Ce substrat est constitué à 95% de matière sèche, il semble donc logique de se tourner vers des technologies de digestion anaérobie en voie sèche puisqu'elles permettent la digestion de la matière pour des teneurs en matière sèche supérieures à 15% (Li et *al.*, 2011). De plus ce choix présente des avantages, tels que la possibilité de traiter plus de substrat par volume de réacteur d'où des réacteurs moins coûteux. La technologie batch semble plus adaptée aux exploitations agricoles individuelles par rapport aux technologies continues. Effectivement, les entrées et sorties moins fréquentes de matière facilitent la gestion du procédé et les réacteurs sont moins chers.

Afin d'améliorer la digestion anaérobie de la biomasse lignocellulosique, l'objectif principal des prétraitements doit être la délignification (Monlau et *al.*, 2012). Pour ce faire, les pourritures blanches sont particulièrement intéressantes. Ces basidiomycètes ont, en effet, un système enzymatique unique qui leur confère une bonne efficacité pour oxyder la lignine. Les enzymes lignolytiques les plus répandues sont la laccase, la lignine peroxydase (LiP) et la manganese peroxydase (MnP). Ces dernières possèdent un noyau hémique alors que la laccase contient du cuivre. La présence nécessaire de manganèse pour le bon fonctionnement de la manganèse peroxydase peut également être relevée. Les champignons peuvent être cultivés en milieu solide ou en milieu liquide mais les enzymes lignolytiques sont plus efficaces en milieu solide et les coûts sont moindres, notamment grâce aux réacteurs moins complexes. Finalement, la culture en milieu solide va être compatible avec la méthanisation voie sèche. Ce type de culture a donc été retenu pour les prétraitements fongiques du projet Stockactif.

L'une des problématiques lors d'un prétraitement fongique concerne la perte de matière (sous forme de CO_2) par respiration des champignons. Cette perte de matière peut limiter la rentabilité du procédé. En théorie, si les pertes de matière sont constituées exclusivement de lignine, la production de méthane par

digestion anaérobie de la paille prétraitée va augmenter. En revanche, si les pertes sont constituées exclusivement de sucres, la production de méthane sera diminuée car il s'agit du substrat de la méthanisation. Dans la réalité, la lignine et les sucres sont dégradés et, selon les proportions entre ces deux parties, le prétraitement fongique peut mener à des augmentations ou diminutions de la production de méthane. L'efficacité du prétraitement requiert donc une délignification sélective du substrat.

Pour ce faire, il est nécessaire de choisir une souche fongique efficace et d'en optimiser les conditions de culture, ce qui constituait deux objectifs spécifiques de cette thèse. Ce travail a été réalisé en étroite collaboration avec l'UMR BBF de l'INRA de Marseille (Figure 1). Le dernier objectif spécifique de la thèse, consistait à accroître l'échelle du prétraitement et de la méthanisation afin de caractériser la Digestion Anaérobie Voie Sèche (DAVS) de la paille prétraitée. La question scientifique sous-jacente commune aux objectifs spécifiques était : comment et dans quelles proportions le prétraitement fongique impacte-t-il la digestion anaérobie de la paille de blé ? Pour répondre à cette question, non seulement la digestion anaérobie des pailles prétraitées devait être étudiée mais les modifications de la paille prétraitée devaient être caractérisées. Pour cela, la technique de pyrolyse-GC-MS a notamment été utilisée afin de déterminer si elle pouvait permettre de mesurer les modifications de la biomasse après prétraitement et d'évaluer la dégradabilité anaérobie des pailles prétraitées.

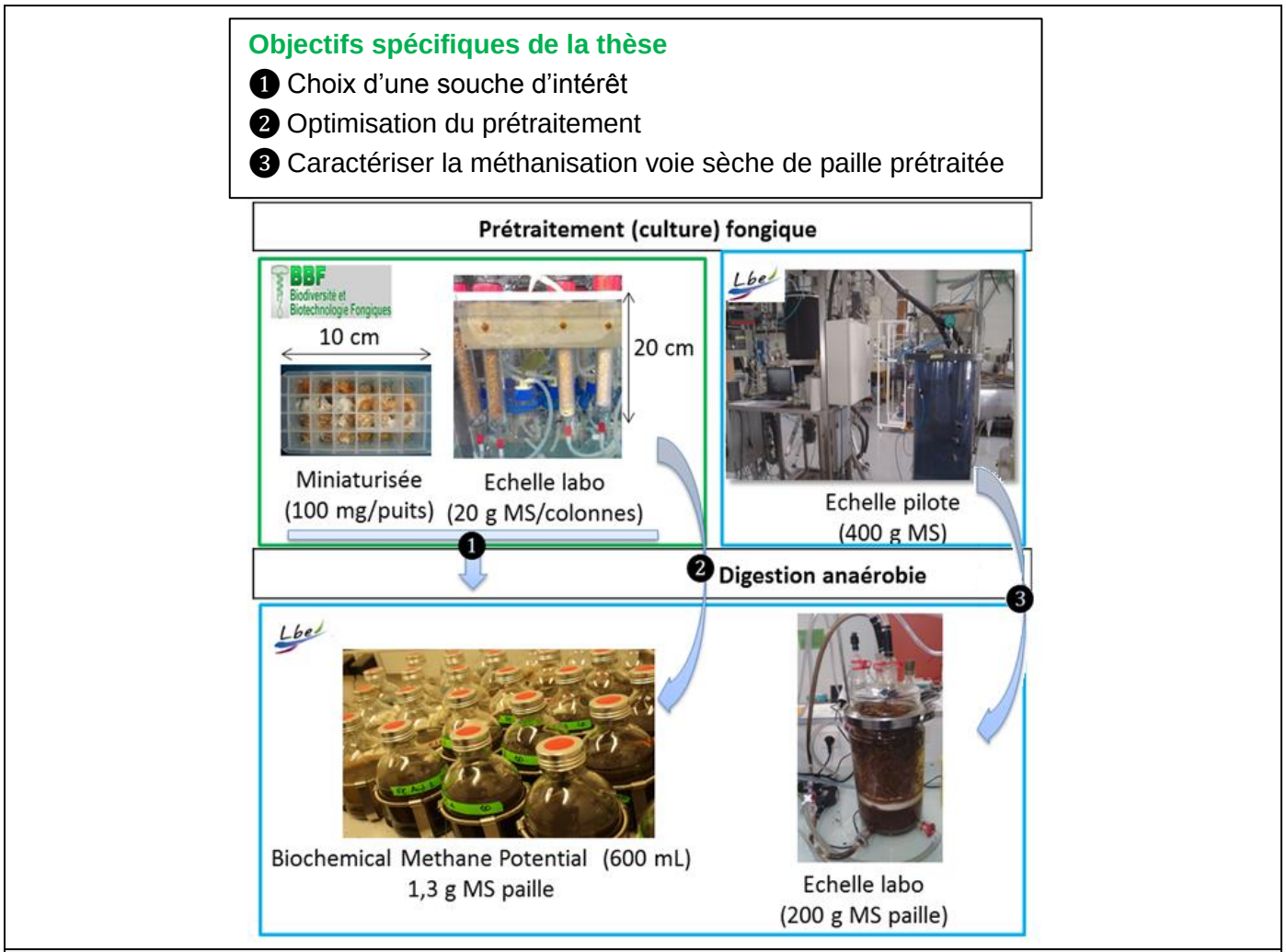


Figure 1. Positionnement des objectifs de la thèse (LBE en bleu) dans le contexte expérimental.

Dans un premier temps, une souche d'intérêt a été identifiée. Un screening sur soixante-trois souches de basidiomycètes a été mené par l'UMR BBF, le critère principal étant les sucres relargués après prétraitement et hydrolyse enzymatique. Suite à cela, douze souches ont été cultivées avec différentes conditions de culture pour étudier l'impact du prétraitement sur la digestion anaérobie (tests BMP, Biochemical Methane Potential). Cela a permis de mettre en évidence l'efficacité de la souche de pourriture blanche *Polyporus brumalis* BRFM 985. Dans le meilleur des cas (en présence de 25 mg glucose/g MS + 2.3 mg de diammonium tartrate/g MS paille), cette souche a mené à une amélioration de la production méthane de 43% par gramme de matière volatile comparée à une paille témoin non inoculée. Après prise en compte des pertes de matière durant le prétraitement, 21% de méthane en plus par gramme de matière sèche (MS) initiale (avant prétraitement) ont été constatés. Il s'agit de la meilleure amélioration obtenue à ce jour pour des pailles et biomasses herbacées, notamment en raison d'un faible nombre d'études sur le sujet (Rouches et *al.*, 2016).

D'autre part, les conditions de culture ont fortement influé sur la méthanisation des pailles, l'efficacité du prétraitement étant fonction de la souche fongique, du substrat utilisé mais aussi des conditions du prétraitement (Wan and Li, 2012). L'addition de glucose lors du prétraitement a plus particulièrement été étudiée en raison d'un manque de clarté de la littérature sur son rôle et de l'incapacité de certaines souches à croître en l'absence de supplémentation en glucose. De plus, une tendance à impacter négativement la production de méthane a été constatée en présence d'un fort ajout de solution nutritive (200 mg de glucose/g MS paille + 18.4 mg de diammonium tartrate/g MS paille). Différentes quantités de glucose ont alors été testées lors du prétraitement (0, 50, 200, 400 mg de glucose/g MS paille) et la composition des pailles prétraitées a été analysée. Deux souches ont été étudiées afin de déterminer si les mécanismes étaient souche-dépendants : *P. brumalis* BRFM 985 et *Trametes menziesii* BRFM 1369. Les conditions de température et de durée du prétraitement étaient fixées à 25°C durant 12 jours. La délignification par les champignons a diminué avec des ajouts croissants de glucose (entre 50 et 400 mg/g MS). La consommation des hémicelluloses demeura assez stable quelle que soit la condition testée. La consommation de la cellulose peut être influencée par la quantité de glucose ajoutée lors du prétraitement d'une façon souche dépendante. Cette consommation a augmenté avec l'apport de glucose pour *T. menziesii* BRFM 1369 alors que *P. brumalis* BRFM 985 a peu consommé de cellulose en présence de glucose. Il est possible que les cellulases de cette souche aient été inhibées par l'ajout de glucose (Moreira et *al.*, 1997). L'ajout de glucose pourrait accroître la durée du métabolisme primaire durant lequel il y a peu de délignification accompagnée d'une consommation des sucres facilement accessibles (Shi et *al.*, 2014).

Etant donné l'importance des conditions de culture, une optimisation du prétraitement avec la souche *P. brumalis* BRFM 985 a été menée afin de maximiser la production de méthane et de comprendre l'influence des conditions de culture sur le substrat et sa méthanisation. Un plan d'expérience de Doelhert a été réalisé par l'UMR BBF, consistant à tester la Masse Humide (MH)/MS paille initiale en début de prétraitement (de 2.1 à 4.5 g/g), la durée (entre 10 et 20 jours) et la température de culture (entre 20 et 30°C). Finalement, l'ajout ou non d'une solution de métaux (Cu^{2+}, Fe^{2+}, Mn^{2+} à raison de 0,9 mM/g MS paille), supposée favoriser l'activité des enzymes lignolytiques (Salvachúa et *al.*, 2013), a également été testée. *P. brumalis* BRFM 985 pouvant croître sans glucose et le glucose représentant un coût additionnel potentiellement important, le plan d'expérience a été mené en l'absence de supplémentation en glucose.

Après méthanisation des pailles prétraitées, les surfaces de réponses de la production de méthane en NmL de CH_4/g MS initiale (prise en compte des pertes de matière) ont été tracées. Celles-ci ont montré un fort impact positif de l'ajout de métaux lors du prétraitement sur la méthanisation (parfois plus de 30 NmL/g MS initiale (i) supplémentaires avec métaux). La durée de culture a également fortement influencé la production de méthane avec une production minimisée aux environs de 15 jours de prétraitement et maximisée (ou presque) pour de courtes durées de culture (≈11 j) (Table 1). En présence de métaux, la meilleure production de méthane a été obtenue pour de longues durées de culture (≈20 j).

Table 1. Extrema pour les surfaces de réponses de la production de methane estimée par tests BMP.

Metaux	Durée *(j)*	MH/MS	Température *(°C)*	BMP maximum calculées *(NmL CH_4/g MSi)*	BMP minimum calculées *(NmL CH_4/g MSi)*
Oui	11,37	4,00	27,84	206	
Oui	19,99	3,22	25,04	213	
Non	10,31	3,74	25,85	185	
Non	19,54	2,98	22,91	169	
Oui	13,48	3,18	19,31		142
Non	14,92	3,81	19,42		141

L'humidité initiale de la paille ainsi que la température de culture se sont révélées moins influentes (moins de 10 NmL/g MS i, avec les autres conditions fixées). A l'avenir, il est donc possible de fixer ces paramètres pour approfondir l'étude de la durée de culture pour laquelle le domaine expérimental trop restreint n'a pas permis de mettre en évidence l'optimum de la production de méthane. En outre, la meilleure amélioration constatée après prise en compte des pertes de matière n'était que de 5% (contre 20% précédemment). Il est possible que l'ajout d'une légère quantité de glucose lors de l'étape de sélection précédente ait été un facteur déterminant pour l'efficacité du prétraitement (possible inhibition des cellulases de *P. brumalis*).

Afin de mieux expliquer l'influence des paramètres du prétraitement sur la production de méthane, d'autres surfaces de réponse ont été tracées à partir des caractérisations de matière effectuées par l'UMR BBF. L'addition de métaux a permis en moyenne 5% MS de pertes de lignine en plus, d'où leur impact positif sur la production méthane. En revanche les métaux n'ont pas influencé les pertes totales de

matière. Celles-ci augmentaient avec la durée du prétraitement. Pour de longues durées de culture, à pertes de matières égales, la proportion de lignine consommée était plus importante en présence de métaux que sans. Ceci expliquerait le maximum observé pour de longues durées de culture avec métaux.

D'autre part, une importante et rapide digestibilité anaérobie pour la biomasse fongique a été montrée. Quelques mesures de biomasse fongique sur la paille prétraitée démontrent une très légère influence positive de la biomasse fongique sur la production de méthane des pailles prétraitées. De plus, le rendement glucose après hydrolyse enzymatique a été positivement corrélé à la production de méthane après prétraitement mais etait surtout négativement corrélé à la teneur en lignine de la paille ($R^2 = 0,95$).

Des pyrolyses-Chromatographies Gazeuses-Spectrophotometries de Masse (py-GC-MS) ont en outre été effectuées à l'UMR EcoSYS avec diverses pailles prétraitées. Cette technique semi-quantitative est considérée comme rapide, sensible et reproductible (Galletti and Bocchini, 1995). De plus, elle fournit des informations sur des composés divers (protéines, lignine, sucres…). En utilisant une large gamme de teneur en lignine, des études ont établi une corrélation entre la lignine Klason et la lignine déterminée par py-GC-MS (Alves et *al.*, 2006; Fahmi et *al.*, 2007; Ross & Mazza, 2011).

L'étude du mycelium de *P. brumalis* BRFM 985 par Py-GC-MS a montré qu'il était majoritairement constitué de sucres et de protéines d'où sa bonne dégradabilité anaérobie. Le glucose a été identifié comme un élément majoritaire des polysaccharides du mycelium. Des hypothèses sur la composition en acides aminés de *P. brumalis* BRFM 985 ont également pu être formulées, avec une part supposée conséquente de phénylalanine et tyrosine. Cette hypothèse provenait de la présence importante de toluène et styrène dans le pyrolysat du mycelium qui peuvent dériver des acides aminés évoqués. L'aire relative de ces composés sur les pyrolysats des pailles prétraitées a été corrélée à la biomasse fongique déterminée par qPCR de ces pailles (R^2 entre 0,7 et 0,8). Cette corrélation a montré que la Py-GC-MS pourrait permettre une estimation de la biomasse fongique sur des pailles de blé prétraitées avec différentes souches de pourritures blanches dans des conditions identiques.

Au regard de l'absence de corrélation entre la lignine (LIG) déterminée par Py-GC-MS avec la lignine obtenue par la technique plus usuelle d'hydrolyse acide (LIG NREL), il semblait pertinent d'étudier le ratio polysaccharides/lignine (PS/LIG) déterminé par Py-GC-MS pour le comparer à celui obtenu par hydrolyse acide (PS/LIG NREL). Cependant, ces deux ratios n'étaient pas corrélés. En revanche, le ratio PS/LIG obtenu par Py-GC-MS était corrélé à la production de méthane des pailles prétraitées ($R^2 = 0,6$), de même que PS/LIG NREL ($R^2 = 0,6$). Cela montre que le ratio PS/LIG pourrait être un bon indicateur de la dégradabilité anaérobie de pailles de blé prétraitées dans les mêmes conditions avec différentes souches. Cet indicateur pourrait être déterminé par Py-GC-MS et permettrait une pré-sélection des souches. D'autres expériences sont requises pour savoir dans quelles mesures ce ratio pourrait être utilisé avec d'autres types d'échantillons prétraités fongiquement (autres substrats, autres conditions de culture…).

Finalement, la Digestion Anaérobie par Voie Sèche (DAVS) de la paille de blé a été étudiée en réacteurs batch de 6 L. Cela a permis un accroissement de l'échelle d'étude de la digestion anaérobie qui était jusqu'alors effectuée en voie liquide en fiole de 600 mL (tests BMP). Dans une première expérience, le ratio substrat/inoculum (S/I) adéquat a été déterminé afin de traiter le plus de substrat possible tout en évitant les problèmes d'acidification. L'acidification provient d'une accumulation d'Acides Gras Volatils (AGV) suite à un apport de substrat trop conséquent. Une forte acidification peut mener à une longue période d'inhibition de la production de méthane car les microorganismes méthanogènes fonctionnent préférentiellement à des pH neutres. Le choix d'un ratio S/I adéquat permet donc de d'assurer une production de méthane maximale par volume de réacteur. Pour la paille de blé, un S/I compris entre 2 et 4 (en matières volatiles) semble bien adapté à la DAVS. Alors qu'en voie liquide des ratios AGV/alcalinité inférieurs à 0.6 correspondent à des réacteurs stables, cette limite pourrai être inadaptée à la DAVS. Il a été observé que pour un ratio AGV/alcalinité inférieur à deux et avec une concentration d'AGV dans les lixiviats inférieure à 10 g/L, la production de méthane pouvait être rétablie après une acidification.

Dans une seconde expérience, l'échelle du prétraitement a été accrue (de 20 g MS à 400 g MS) et bien que le substrat utilisé fût toujours stérile, le réacteur de prétraitement ne l'était pas. En l'état des connaissances actuelles, le travail à grande échelle dans des conditions stériles seraient, en effet, coûteux et peu pratique. Après plusieurs tentatives, de la paille prétraitée avec *P. brumalis* BRFM 985 a été obtenue, celle-ci ne présentait pas de contaminations visibles à l'œil nu mais de larges zones non prétraitées. Cette paille prétraitée a été digérée en DAVS, ce qui a montré une légère amélioration de sa biodegradabilité par comparaison aux témoins ainsi qu'un démarrage de la méthanisation facilité (peu d'acidification). Cependant, après prise en compte des pertes de matière (de l'ordre de 20%), le prétraitement s'est révélé inefficace probablement en raison de conditions de culture non optimales.

Pour conclure, il faut rappeler qu'une bonne amélioration de la production de méthane après prise en compte des pertes de matières lors du prétraitement a été obtenue (de l'ordre de 20%). Cette amélioration provenait d'une délignification importante par *P. brumalis* BRFM 985 ainsi que d'une certaine conservation de la cellulose en présence d'une légère quantité de glucose (25 mg/g MS). Finalement, une diminution importante de la cristallinité de la cellulose (≈ 50 % de cellulose cristalline en moins) a également été mesurée dans ces conditions. Une telle diminution est reconnue dans la littérature comme un facteur favorisant la production de méthane même si l'impact des pourritures blanches sur la cristallinité de la cellulose est peu caractérisé dans la littérature (Rouches et *al.*, 2016).

Le prétraitement par pourritures blanches est largement étudié pour la production de bioéthanol mais il a été peu caractérisé pour la production de biométhane. Par exemple, bien que constituant des points importants pour l'efficacité du prétraitement, les conditions de culture ou les pertes de matières sont rarement discutées dans les études dédiées. Les travaux de cette thèse soulignent: (i) la nécessité de prendre en compte les pertes de matière durant le prétraitement pour en évaluer son efficacité ; (ii) l'importance de l'étude des conditions de culture pour l'évaluation des souches fongiques ; (iii) le besoin de recherche pour favoriser le prétraitement fongique d'un substrat en conditions non stériles. Diverses études restent nécessaires pour envisager une exploitation industrielle du procédé, comme par exemple l'évaluation des coûts d'un prétraitement fongique à grande échelle.

Finalement diverses perspectives plus larges peuvent être envisagées. La combinaison d'un prétraitement fongique avec un prétraitement non-biologique pourrait permettre un gain de temps par rapport à un prétraitement fongique seul ainsi qu'un prétraitement plus économique par rapport à un prétraitement non- biologique seul. Certains auteurs rapportent d'ailleurs une amélioration de la délignification suite à une telle combinaison de prétraitement (Wan and Li, 2012). La combinaison de plusieurs souches pourrait également avoir un effet synergique sur la délignification (Sundman and Näse, 1972). Finalement, l'étude d'autres substrats (comme le fumier de cheval) ou d'autres usages (comme la production de biohydrogène) après prétraitement fongique représente également une importante perspective.

Introduction

"Human influence on the climate system is clear, and recent anthropogenic emissions of greenhouse gases are the highest in history. Recent **climate changes** have had widespread impacts on human and natural systems." Global warming increases the intensity and frequency of extreme climatic events (heat waves, floods, storms). Without modification of our lifestyles, ecosystems will be deeply modified leading notably to food and water shortages and diseases development. Use of fossil fuels releasing greenhouse gases has a major impact in those phenomena (Pachauri et *al.*, 2014). Moreover with petrol, there is a frequent reliance to foreign energy and its depletion implies regularly rising prices, recourse to renewable energy is also interesting from an **economical** point of view (Mohan et *al.*, 2006).

To be sustainable and avoid increasing **starvation**, energy production must favor substrates that do not compete with food production; as with increasing population, global food production must be very efficient although facing the diminishing of fertile farmlands (erosion and degradation of **soils**, urbanization…) (Ehrlich et *al.*, 1993). Consequently, emerging **second generation** biorefineries are based on non-edible lignocellulosic substrates (Frigon and Guiot, 2010). This approach also allows a greater net energy generation per area of land (Hallac et *al.*, 2009). Valorization of materials previously considered as wastes becomes possible. **Waste management** is also an important concern as it can be illustrated by the legislation for wastes reduction of the European Union (Directive n° 2008/98/CE).

Thus, **lignocellulosic biomass** (LB) is a choice substrate for energy production because it is renewable, widely available and rich in sugars (55-75% dry matter) (Wan & Li, 2012). Crop residues, such as straw, must be preferred since they generate a limited competition for land use with food. Lignocellulosic biomass is principally constituted by cellulose, hemicelluloses (carbohydrates) and **lignin** (phenols). Lignin hydrolysis is difficult (Grabber, 2005; Karunanandaa et *al.*, 1995; Monlau et *al.*, 2013). Consequently, carbohydrates are less accessible for energy production owing to interactions and linkages with lignin forming the Lignin Carbohydrates Complex (LCC). Furthermore, other parameters such as the cellulose crystallinity increase cell-wall robustness and restrain the biodegradation (Monlau et *al.*, 2012a). Several pretreatments of lignocellulosic biomass have been studied to overcome those bottlenecks (Shah et *al.*, 2015). The main goal is to break down linkages between polysaccharides and lignin to increase their conversion yield into renewable fuels production.

Pretreatments, especially physical (grinding…) and thermo-chemical (acid, steam explosion…) constitute an expensive step representing a significant barrier to the marketing of bioenergy production processes. They also can generate large amounts of wastes. Moreover, the European Directive 2009/28/EC requires environmental performance for renewable fuels and it is the whole life cycle that needs to be investigated. Although less studied, biological pretreatments (fungi, enzymes, etc.) have significantly less inconvenience. In particular, the use of fungal pretreatments enables economical gain notably by low energy (ambient

temperature) and chemicals requirements.. They are also less expensive than enzymatic treatments necessitating enzymes production and extraction steps. Furthermore, fungi have environmental benefits with low inputs and outputs (inhibitors and wastes). Among fungi, White-Rot Fungi (**WRF**) are considered as the most efficient microorganisms to delignify a substrate. However, fungal pretreatments are not only strain and substrate dependent but culture conditions also highly influence their efficiency (Wan and Li, 2012). Moreover, they require several weeks for the establishment of a sufficient fungal population to significantly alter the substrate.

To circumvent this inconvenient and as high organic matter losses can occur during storage period (Herrmann et *al.*, 2011), the idea to use the storage period to pretreat the substrate is the object of the ANR **STOCKACTIF project**. This project concerns the growth of ligninolytic fungi under solid-state fermentation (**SSF**). The main advantage is lower costs with SSF than with liquid culture (less aeration, heating, agitating) (Tian et *al.*, 2012). The Stockactif project is motivated by a potential receptivity to innovation for industries owing to the relatively recent interest towards lignocellulosic biomass. The desired fungal pretreatment must, among others, limit the hydrolysis of carbohydrates polymers (cellulose and hemicelluloses). Carbohydrates are the substrate for fuels production and their consumption by fungi would lead to a lower biofuel production. **Project challenges** are to preserve a maximum of "biomass use potential" (bioethanol, biogas and building blocks); to identify reproducible storage conditions and to promote the industrial feasibility (cost minimization, etc.).

Several **research and industrial partners** took part in this project (Figure 2). BBF UMR (Unité Mixte de Recherche Biodiversité et Biotechnologie Fongiques), INRA of Marseille, was implied in all aspects concerning fungal culture. FARE UMR (Fractionnement des Agroressources et Environnement), research unity of INRA in Reims studied the bioethanol production from fungal pretreated miscanthus (a bioethanol dedicated culture). LBE UR (Unité de Recherche Laboratoire de Biotechnologie de l'Environnement), or INRA of Narbonne, worked on anaerobic digestion of fungal pretreated wheat straw (particularly through this thesis). To better characterize the structure and composition of the pretreated wheat straw, pyrolysis-GC-MS (Gas Chromatography-Mass Spectrophotometry) and 3D spectrophotometry technics were used thanks to ECOSYS UMR (Ecologie fonctionnelle et écotoxicologie des agroécosystèmes), INRA of Grignon and the company Envolure (Montpellier), respectively. In order to favor industrial feasibility, IATE UMR (Ingénierie des Agro-polymères et Technologies Émergentes), INRA of Montpellier, studied the grinding of fungal pretreated biomass and the association Solagro (Toulouse) led a technico-economical study (potential costs, existing industrial processes…). Finally two industrial partners will allow the realization of an industrial trial (two tons of wheat straw) at the end of the project (March 2016). It will be carried out by Vivescia, a large agricultural cooperative of Champagnes-Ardennes and Groupe Soufflet, an international society working on solid-state fermentation. Vivescia will organize the large scale anaerobic digestion whereas Soufflet will pretreat straw for the industrial trial.

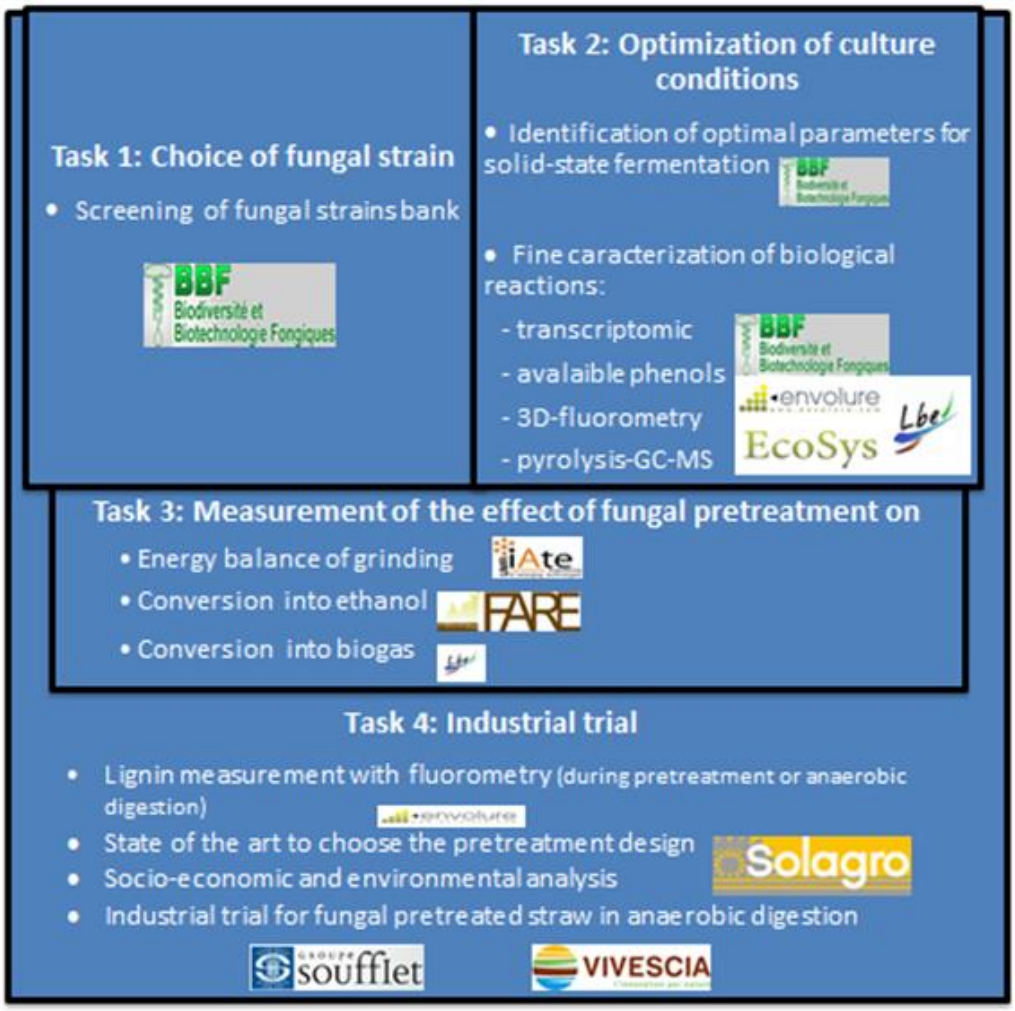


Figure 2. Scheme of the ANR Stockactif project, main tasks and main associated partners.

Anaerobic digestion consists in the degradation of organic matter to biogas (mainly CO_2 and CH_4) thanks to microorganisms and presents several advantages. Among them, the final solid residue (called digestate) can often be valorized as fertilizer (rich in available N, P, K) (Tambone et *al.*, 2010) and methane produced has several applications (heat, electricity, biofuel, injection in gas grid). Methane is generally burnt to produce heat and be converted in electricity thanks to an Internal Combustion Engines. The combustion releases CO_2 that will not increase greenhouse effect as the carbon has been recently removed from the atmosphere by plant uptake, to be soon released in the atmosphere by methane use. Methane is a storable energy that can be used for fluctuating demands. Compared to the majority of liquid biofuels in use today (biodiesel and bioethanol), biogas production from lignocellulosic residues has far better performance regarding life cycle emissions, energy recovered per hectare and energy efficiency (ratio of energy produced to energy input for the production and conversion of biomass) (Börjesson and Mattiasson, 2008). In term of waste management, diverse advantages are also reported such as low odor emissions, effective pathogens removal and valorization of organic matter (Ward et *al.*, 2008). However, some precautions must be taken for the exploitation of anaerobic digesters such as operators training or reactor tightness. It can also be difficult to provide the important investment capacity required or to ensure a lasting wastes supply (ADEME, 2011).

Solid State Anaerobic Digestion (SSAD), working with more than 15% Total Solid, is particularly adapted to the digestion of dry crop residues such as straw. Farm SSAD processes, less expensive and simpler (Li et *al.*, 2011), are at present facing a fast development in France.

This PhD work focusses on the anaerobic digestion of fungal pretreated **wheat straw**. The substrate chosen for anaerobic digestion production is wheat straw since it is: i) an experimental model because the composition of the organic matter (VS) is representative of herbaceous biomass (Vassilev et *al.*, 2012); ii) the main crop residues in Europe and the second worldwide (Talebnia et *al.*, 2010); iii) a biomass that can overcome the problem of feedstock seasonality in biogas plants.

The main parameter to characterize a substrate for anaerobic digestion is its Biochemical Methane Potential (**BMP**) value. This value is obtained with BMP tests that consist to anaerobically digest the substrate under optimal conditions in order to determine the maximal methane production of this substrate: the BMP value. BMP tests allow to compare lot of different conditions but they are not sufficient to predict full-scale performance (Carlsson et *al.* 2012). Anaerobic digestion with higher substrate amount in conditions closest to industrial processes is required to validate and transpose results to higher scale. It is especially the case when Solid State Anaerobic Digestion (SSAD) is applied. Both BMP tests and study in **SSAD reactors** were carried out on fungal pretreated wheat straw in the current work. This work was realized in close collaboration with several partners; and in particular, BBF team, since most pretreatments were realized by Simeng Zhou, a PhD student from BBF (Figure 2).

Three **main applied objectives** were fixed in link with the project tasks (Figure 2):

i) choice of an efficient fungal strain based on anaerobic degradability of pretreated straws (selection step);

ii) with the retained strain, determination of fungal culture conditions maximizing methane production from the pretreated substrate (optimization step);

iii) to provide necessary elements for scale-up (industrial trial) by studying SSAD of pretreated straw.

The **scientific question** common to the three applied objectives was: how and in what proportion do fungal pretreatments impact anaerobic digestion of wheat straw? To answer this question, not only anaerobic digestion of pretreated straws must be investigated but it is also necessary to characterize wheat straw modifications (composition, structure…). For this purpose, it was notably investigated if Pyrolysis-GC-MS technic can provide information to study anaerobic degradability of pretreated straws.

The state of the art, in Chapter 1, particularly emphasizes substrate modifications by white-rot fungi susceptible to improve its anaerobic digestibility. Results chapters 3, 4 and 6 directly aimed to answer to applied objective whereas in Chapter 5 it was attempted to use the py-GC-MS characterization technic to assess pretreatment efficiency. Material and methods specific to each results chapter are detailed in the corresponding chapters whereas global experimental strategy with most common methods are presented in Chapter 2. Finally, a general discussion and expanded prospects are provided in Chapter 7 and Chapter 8, respectively.

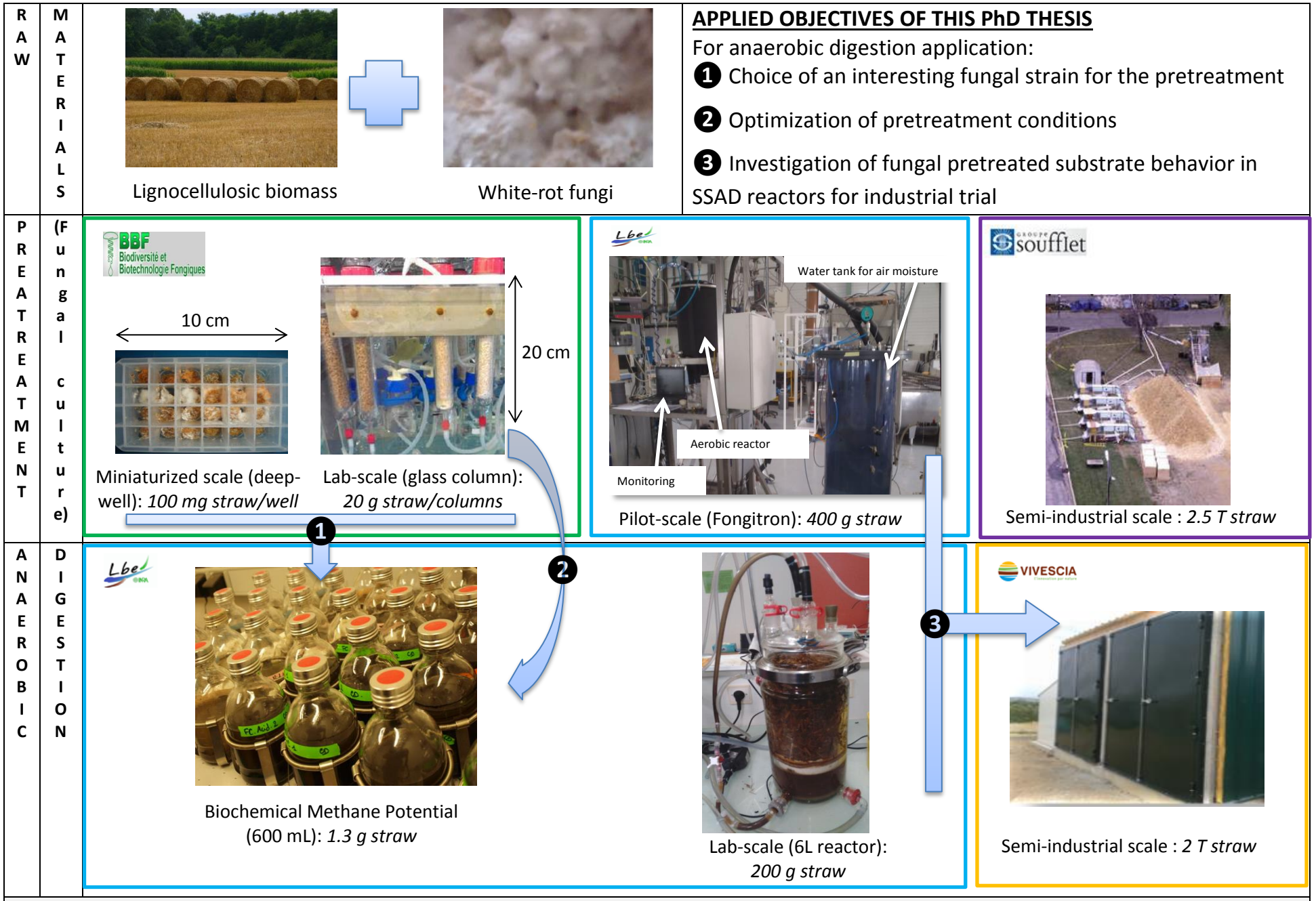


Figure 3. Study of fungal pretreatment for wheat straw anaerobic digestion in Stockactif project: different scales of processes and applied objectives of this PhD thesis.

Chapter 1.

Literature review

This chapter provides, firstly, general information about anaerobic digestion with a focus on solid-state anaerobic digestion. Lignocellulosic residues are also defined (availability, composition, energy use). Then, overview of lignocellulosic pretreatments for anaerobic digestion is presented with a special emphasizes on biological pretreatments and white-rot fungi (WRF) in particular (enzymatic mechanisms and culture parameters). Finally, a detailed bibliographic study on anaerobic digestibility improvement using WRF was carried out (and published in *Renewable & Sustainable Energy Reviews*, 2016: 59, 179-198). It focuses on quantitative aspects, e.g. methane improvement, but also on qualitative aspects, i. e. lignocellulosic structures modifications. The last part of this chapter is dedicated to Pyrolysis-Gas Chromatography-Mass Spectrophotometry to study lignocellulosic biomass (principle, relevance…).

Table of content

1.1 Anaerobic Digestion

Anaerobic digestion is a biological reaction encountered in all environments where there is organic matter in absence of oxygen, and where the physicochemical conditions are compatible with living microorganisms: marshes, rice fields, intestines, etc. (Moletta, 2008)

1.1.1 A four steps biological reaction

Anaerobic digestion (AD) can be divided in **four main steps**, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 1-1), each one involving a specific group of microorganisms (blue rectangles on Figure 1-1). Firstly during the hydrolysis step, complex organic molecules (proteins, lipids and carbohydrates) are hydrolyzed into soluble monomers (simple sugars, amino acids, and fatty acids).

Hydrolytic bacteria secrete extracellular enzymes. Even if population doubling times reach only few hours, this step is generally considered as rate-limiting for solid wastes (Moletta, 2008). Substrate accessibility to enzymes can be difficult, especially for lignocellulosic substrates (Taherzadeh and Karimi, 2008). Thus, improvement of this accessibility can be required (pretreatments, mixing, etc.).

Acidogenesis occurs after hydrolysis or in parallel, fermenting bacteria absorb products of hydrolysis and convert them into Volatile Fatty Acids (VFA), alcohols, hydrogen and carbon dioxide. In steady state anaerobic digestion, main products are acetate, carbon dioxide and hydrogen that can directly be used by the methanogens. Acidogenesis is rapid, even the fastest step for degradation of complex organic matter in liquid phase digestion (Merlin Christy et *al.*, 2013). Thus, it can lead to acids and/or hydrogen accumulation. Those products may be responsible for anaerobic digestion failure inhibiting acetogens and methanogens if not enough numerous.

Hydrolytic and fermenting bacteria are obligate or facultative anaerobes. Genus of implied bacterias are numerous and depend on operating conditions (substrate, pH, temperature, etc.): *Clostridium*, *Bacillus*, *Pseudomonas*, etc. (Moletta, 2008)

During **acetogenesis** takes place the conversion of the acidogenic products (mainly VFA) into acetic acid, hydrogen and carbon dioxide. Syntrophic acetogens are obligate hydrogen-producing bacteria but require low hydrogen partial pressure and therefore a syntrophic association with hydrogen consuming methanogens is required. They are slow growing (few days of population doubling time). Then, homoacetogens produce acetate ($C_2H_3O_2$) by cleavage of organic molecules or by reduction of CO_2 with H_2. Corresponding genus are: *Syntrophomonas*, *Syntrophococcus*, *Butyribacterium*, *Acetoanerobaiuum*, etc.

Finally, methane formation occurs during **methanogenesis** thanks to *Archaea* microorganisms (*Methanohalobium*, *Methanocaldococcus*, etc.). They are obligate anaerobes and can be divided into acetoclastic and hydrogenophilic methanogens. Their population doubling times are between 3 hours to several days. As they grow much slower than acidogenic bacteria, an imbalance in the process can occur,

leading to acidification and finally, ending the anaerobic digestion (Vavilin and Angelidaki, 2005). Hydrogenophilic methanogens use specifically CO_2 and H_2 and live in association with other fermentative microorganisms. The consumption of H_2 gives them a central role in the whole reaction equilibrium. Acetoclastic methanogens have several substrates such as methanol, formate, methylamine, CO_2 and H_2 but the most common is acetate. Words 'methylotroph methanogens' are sometimes employed to designate organisms using methanol and methylamine. Acetoclastic methanogens generate more than 70% of methane produced. They are less resistant to environmental changes than hydrogenophilic (Merlin Christy et *al.*, 2013; Moletta, 2008). Final product of anaerobic digestion, called biogas, is mainly composed of methane (55-75 vol %) and of carbon dioxide (25-45 vol %).

Other minor reactions can take place in stable reactors (Figure 1-1). If sulfate concentrations are sufficient, sulfate-reducing microorganisms (bacteria and *Archaea*) grow faster than methanogens. Sulfate-reducing microorganisms are microaerophiles and produce H_2S. This gas can inhibit methanogens directly or by metals precipitation (essential nutrient for them). Without sulfate, sulfate-reducing microorganisms can oxidize organic acids and alcohols. Some of them work in facultative syntrophy with hydrogenophilic methanogens. Then, nitrate reduction (final product NH_3) and denitrification (final product N_2) arise with enough nitrogen in the medium.

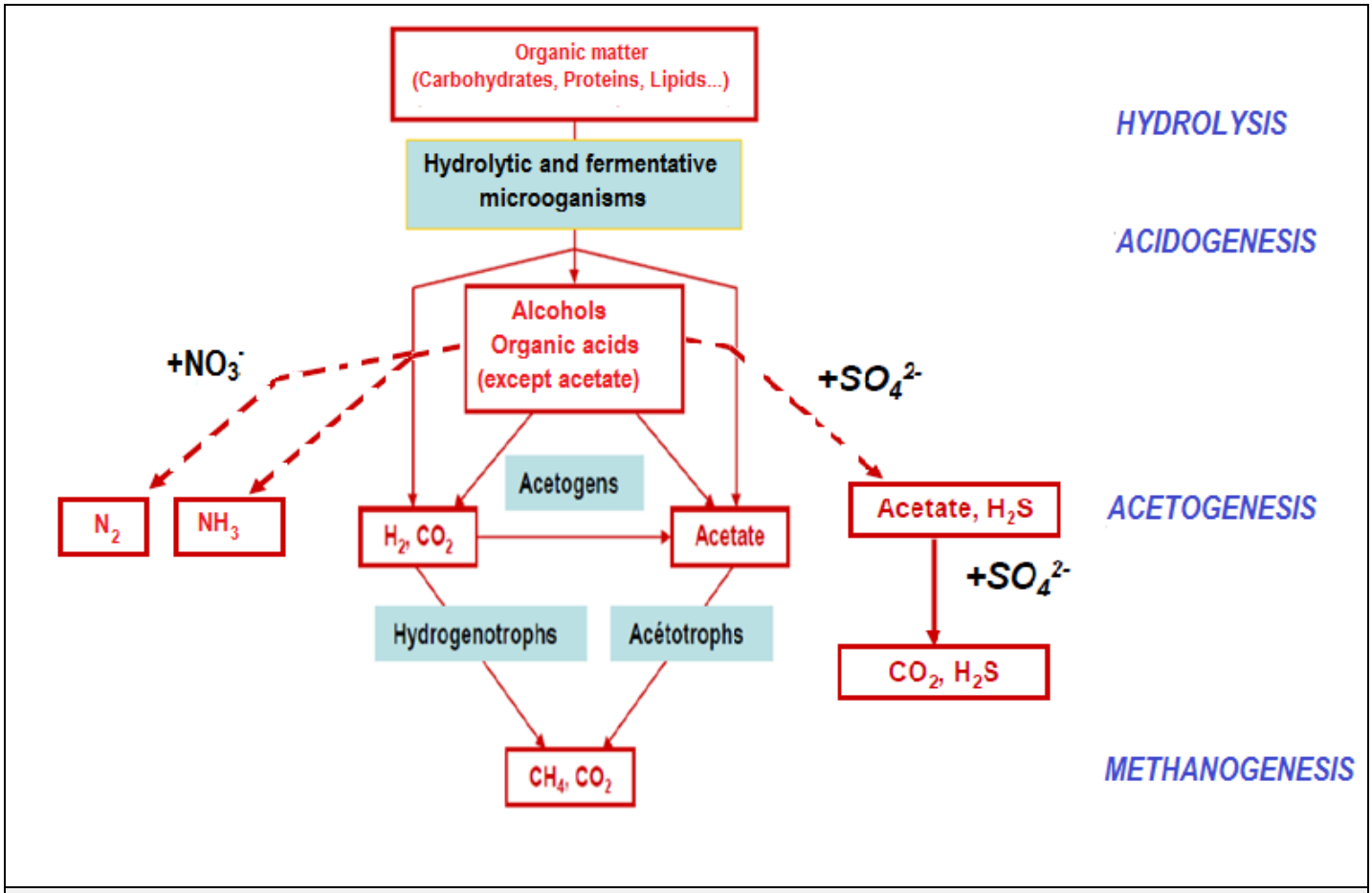


Figure 1-1. Conversion of organic matter to methane by anaerobic digestion.
Solid lines represent the usual pathways in anaerobic digestion whereas dash lines indicate alternative pathways.
(Adapted from Alvarado et *al.*, 2014)

1.1.2 Anaerobic digestion of lignocellulosic crop residues

1.1.2.1 Crop residues: an important resource

To obtain a sustainable energy production from lignocellulosic biomass, only **residues** should be used. They are constituted by woody and non-woody biomasses. Woody biomasses, with their high lignin content, lead to almost no methane production that is why lignocellulosic residues concerned by anaerobic digestion are mainly crop residues issued from agriculture. A part of the biomass that will not lack to another use such as food, feed but also soil fertility can be considered as a residue.

Plants growth implies an uptake of fertilising elements into soil. In natural ecosystems, those elements return to the soil at plants death, creating no soil depletion. In agricultural ecosystems, some plants parts (at least edible one) are exported out of the field and do not return to the soil. In those ecosystems, fertilizers and amendments are generally used and it is possible to take off a certain amount of plants without leading to a soil degradation in terms of organic matter and nutrients (N, P, K…). Organic matter in soil maintains its agronomic value by providing a structure (less sensitivity to erosion, better water circulation, etc.) and a long term nutrients release. From an economical point of view, soil preservation is crucial because without it agricultural yields can drastically decrease. Thus, quantification of lignocellulosic resources available for energy production should not include the **part necessary for the return back to the soil**. However, this part is difficult to estimate as it is a function of soil characteristics (texture, nutrients…) and of agricultural system (crops rotations and requirements, fertilization strategies…) (Castillon et *al.*, 1993; Tomis et *al.*, 2009). In average, it is considered that around 50% of the culture must return to the soil (FranceAgrimer, 2012). The possible uptake would be greater if biomass is used for anaerobic digestion since digestate can be spread to fields leading to a partial restitution of the uptake into the soil. Digestates are significantly different from initial feedstock and their properties are not well known (Tambone et *al.*, 2010). Moreover, there is a lack of study concerning the influence of single feedstock on nutrient contents and speciation in digestates (Möller and Müller, 2012). Thus, uncertainties on possible uptake remain.

Non-woody agricultural residues are principally constituted by gramineous and oleaginous straws. In France, cereal culture concern 60% of arable lands with a majority of wheat. The use and availability of these residues depend of the territorial context and of the agricultural holding (yield, etc.) (FranceAgrimer, 2012; Motte, 2013). Among available straws, i. e. after taking into account the part necessary to soil fertility maintenance, a major part is dedicated to breeding as litter (≈ 54%), minor amounts are incinerated for energy recovery or used as raw material in industry such as chemical, construction or paper industries.

digestion. Sometimes when straw amount is important, the composition is related to straw but substantial modifications occurred (crushed fibers, nitrogen enrichment with urea…) and it can be no more considered as straw. Thus, remaining usable part is important.

In France, more than 4000 kPET (Petroleum Equivalent Ton) per year could be produced from crop residues without soil damage. As it can be observed in Figure 1-2,crop residues are largely under exploited and

represent one of the greatest options for improvement (with manure) among other biomasses usable in anaerobic digestion (waste from food industry…) (FranceAgrimer, 2012). Anaerobic digestion of lignocellulosic residues occurs principally in farm digesters. Organic Fractions of Municipal Solid Waste (OFMSW) can also be considered as lignocelulosic residues (vegetable peelings…). However, they are not included in the Figure 1-2, they are generally valorised even if the valorisation is not always optimal (methane production in landfill…). Moreover, Figure 1-2 shows volumes, lignocellulosic residues have generally a low density and repartition will be changed with a figure showing mass. Finally, forest represents the highest energetical potential but its transformation into methane with anaerobic digestion is not efficient and other energy systems must be chosen.

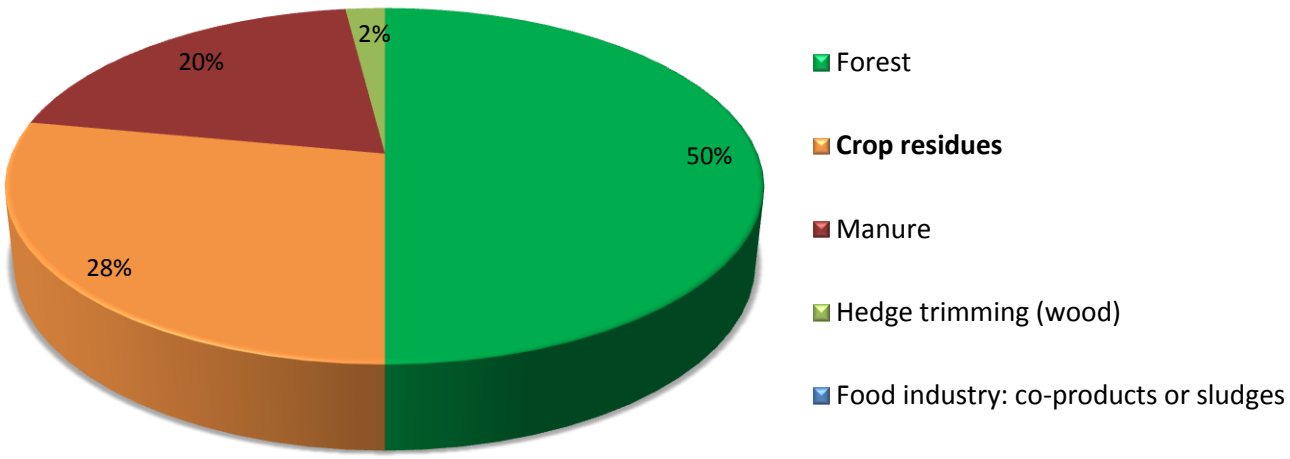


Figure 1-2. Repartition of supplementary volumes available for energy production from biomass.
(FranceAgrimer, 2012)

1.1.2.2 Main constituents of lignocellulosic biomass

1.1.2.2.1 Cellulose

Cellulose, the most abundant LB polymer, is composed of dimeric subunits of D-anhydroglycopyranose (glucose anhydride) connected by β-1,4 glycosidic bonds (Figure 1-3A). These dimers or cellobiose combine in cellulose microfibrils through hydrogen bonds and van der Waals interactions. Cellulose molecules contain between 15 and 15 000 glucose units (chair conformation). The antiparallel arrangement allows the establishment of various hydrogen bonds in a same chain (dotted in the Figure 1-3) (Sun, 2010). H-bonds are also formed between fibers making microfibrils sheets that associate into tubular structures (Figure 1-3B) (Mohan et *al.*, 2006). In plants, the majority of cellulose is under an organized/oriented form and it is called crystalline cellulose. Less structured areas correspond to amorphous cellulose (also called paracrystalline). Various arrangements have been proposed for amorphous and crystalline regions (Figure 1-3C) (Sun, 2010). Amorphous cellulose is more accessible for enzymatic degradation (Sánchez, 2009) and

less hydrophobic. Cellulose accessibility depends also of molecular weight, particle size, degree of polymerization. Those parameters influence surface area and solubility. These elements depend on the plant species, but also on the tissue and plant maturity.

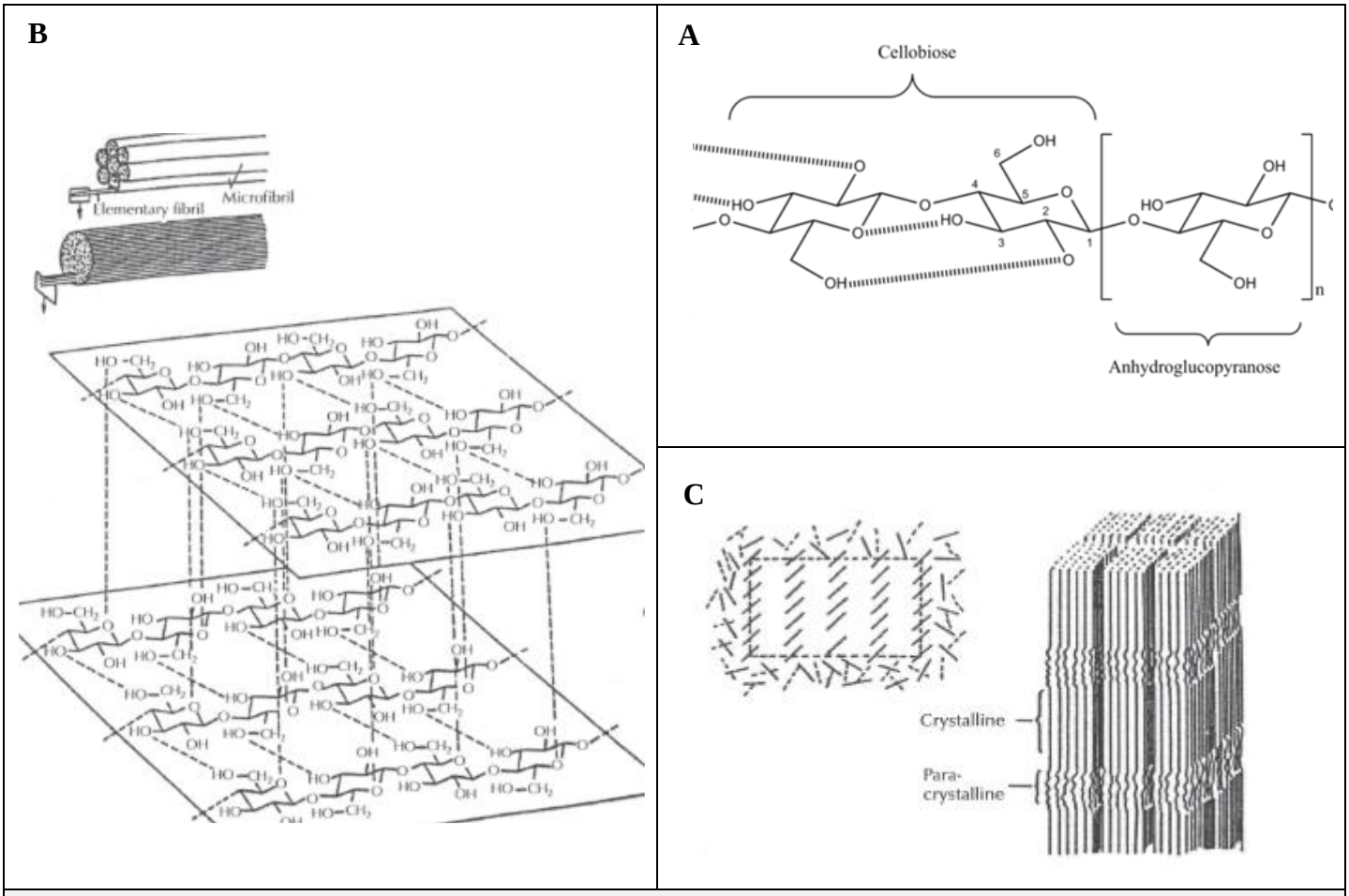


Figure 1-3. Molecular and fibrillary structure of cellulose.
A) Intra-chain H bonds formation between cellobiose subunits of cellulose. B) Fibrillary organization of cellulose due to inter-chain H bonds formation. C) Possible organization between crystalline and amorphous cellulose. (Sun, 2010)

1.1.2.2.2 Hemicelluloses

Hemicelluloses are the second most abundant compound in cereals where they consist mainly of D-xylose for grasses. In all plants, xylose is the main sugar monomer of hemicelluloses. Besides, xylan is the principal component of hemicelluloses from hardwood and crops whereas glucomannan is dominant in softwoods. Hemicelluloses have an amorphous structure (Monlau et *al.* 2013). In straws, other sugars from hemicelluloses are D-mannose, D-galactose, D-glucose, L-arabinose, and acids (uronic, ferulic, glucuronic). These sugars are linked by β-1,4 glycosidic and sometimes by β-1,3 glycosidic bonds (Sanchez, 2009). Hemicelluloses are soluble in basic medium after extraction of pectic substances. The polymer has a lower molecular weight than cellulose since it is composed of 80 to 200 units of hexoses and pentoses. An important part of cereal hemicelluloses are acetylated xylose (Sun, 2010). Hemicelluloses composition varies also with species, tissue and plant maturity.

1.1.2.2.3 Lignin

Lignin is the third most abundant polymer in nature, 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). Ratios between the different lignin monomers vary according to species, maturity, and tissues. Contrarily to woody biomass, the three units are in significant amounts in herbaceous plants, e. g. for wheat straw G/S/H is 49/46/5 (Monlau et *al.*, 2013) (Annex 1). Several linkages exist between those units. Some of them are condensed, i.e. more resistant to degradation. They are classified as carbon-carbon bonds (5–5, β-1, β-5, β-β) or diaryl-ether (4-O-5). Uncondensed linkages are aryl-ether type, in majority β-O-4, but also α-O-4 (Figure 1-4). The molecule organization will affect its hydrophobicity. Lignin starts to dissolve in water at around 180°C under neutral conditions (Kubikova et *al.*, 1996). Its solubility in different pH depends on lignin precursor (p-coumaryl, coniferyl, sinapyl alcohols) (Grabber, 2005). The role of lignin is to give rigidity, impermeability and resistance to plants (microbial attacks, oxidative stress) (Monlau et *al.*, 2013). Thus, it is difficult to degrade, especially owing to important steric hindrance that limits the access of enzymes to the molecule (Boukari et *al.*, 2009). Chandler et *al.* (1980) showed that one percent lignin decreased organic matter digestion of about 3% (Lesteur et *al.*, 2010).

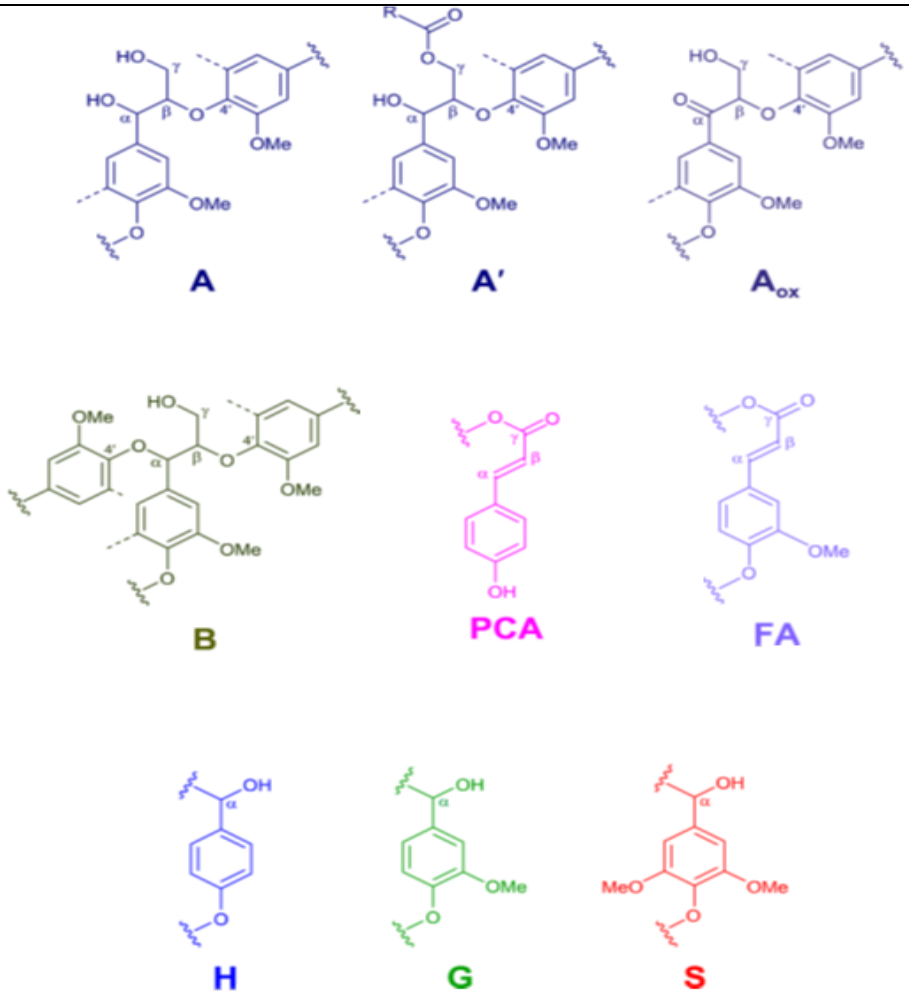


Figure 1-4. Diverse structures present in wheat straw lignin.
A) β-O-4′ alkyl-aryl ethers. A′) β-O-4′ alkyl-aryl ethers with acylated γ-OH. Aox) Cα- oxidized β-O-4′ structures. B) α,β-diaryl ethers. PCA) p-coumarates. FA) ferulates. H) p-hydroxyphenyl units. G) guaiacyl units. S) syringyl units. (Río et *al.*, 2012)

1.1.2.3 Other possibilities for energy recovery from crop residues

Currently, various energy sectors based on the use of LB are developing. It is therefore legitimate to wonder about the advantages and disadvantages of each one. Crop residues can be valorized by combustion (incineration). With wheat straw, recoverable energy amount with combustion is more important than with anaerobic digestion since all organic matter is used (even lignin) (Table 1-1). However with combustion, all organic matter and its nutrients are burnt and do not return to the soil. Moreover, it is a quite expensive process (Kalyuzhnyi et *al.*, 2000). A major technical bottleneck is often reported, it is due to important ash generation leading to material corrosion (Lu et *al.*, 2009). The storability of methane allows a better flexibility than heat generation by combustion (Monlau et *al.*, 2013).

Two other processes emerge for the thermo-chemical conversion of lignocellulose into energy, namely gasification and pyrolysis. Gasification produces a combustible gas mixture by the partial oxidation of biomass under high temperatures (800-900°C). Gas (called syngas) can be then converted to fuel (e. g. methanol) and energy production is high (Table 1-1) (McKendry, 2002a). However, environmental evaluation is not so good even if it would be more environmental friendly than direct combustion. Diverse molecules are generated and lot of them are considered as harmful for human health or with high greenhouse effect (particulate matter, NOx…) (Nguyen et *al.*, 2013). This point must be improved and further research work is necessary in order to apply gasification to biofuel production (Broust et *al.*, 2008).

Flash pyrolysis consists to heat (≈ 500°C) the biomass in absence of oxygen to obtain a liquid product, the bio-oil. Bio-oil would be usable as fuel for diesel vehicles but research efforts are still required to improve its properties, notably to reduce its corrosivity and improve its thermal stability (McKendry, 2002a; S. Wang et *al.*, 2012b). Bio-oils composition and yield can be studied with Pyrolysis-Gas Chromatography-Mass Spectrophotometry (Py-GC-MS) (Mohan et *al.*, 2006). For wheat straw, only 37.6% of the biomass can be converted to bio-oil (Mahinpey et *al.*, 2009) that can explain the little energy production compared to other techniques (Table 1-1).

Wheat straw utilization for bioethanol production leads to a lower energy generation than with anaerobic digestion (Table 1-1). Indeed, hemicelluloses are currently not or low transformed into ethanol whereas very degradable with anaerobic digestion. Furthermore, anaerobic digestion is less sensitive to certain inhibitors (HMF, furfural…) that can be converted to methane after an adaptation period if little concentrated (Barakat et *al.*, 2012). Finally, hydrogen production is possible by anaerobic digestion-like process where methanogenic archaea are inhibited. This process is called dark fermentation. Hydrogen and methane can be produced concomitantly using a two-stage reactor with favorable conditions for methanogens in the second reactor (and unfavorable in the first one). Even if energy yield of biohydrogen seems anecdotic, a combination of small hydrogen amount and methane can be useful to improve combustion (speed and stability) for electricity production or use as biofuel (Kaparaju et *al.*, 2009).

For McKendry (2002a), gasification, pyrolysis and AD are the most likely cost-effective processes. The choice of the technology would be dependent of the requirement since liquid products can easily be transported whereas gas products are best used at the production point.

Table 1-1. Energy production from wheat straw.
(Gaunt and Lehmann, 2008; Adapted from Monlau et *al.*, 2013; Nguyen et *al.*, 2013)

	Bioethanol *(MJ/kg TS)*	CH_4 (Anaerobic digestion) *(MJ/kg TS)*	Combustion *(MJ/kg TS)*	BioHydrogen *(MJ/kg $_{VS\ added}$)*	Bio-oil (Pyrolysis) *(MJ/kg TS)*	Syngas (Gasification) *(MJ/kg TS)*
Wheat straw	6.7	10.2	17.9	0.5	8	14.3

The recovery of high-value molecules, such as phenols, is another possibility for biomass use. Phenols are lignin building-blocks and have interesting antioxidant properties (usable in cosmetic industry…). They could be a valuable by-product from a pretreatment step before anaerobic digestion, especially with biological pretreatment (Sánchez, 2009). Research efforts must continue to easily recover those molecules.

1.1.2.4 A solid substrate adapted to Solid-State anerobic digestion

Crop residues are constituted by water and dry matter (Figure 1-5). Dry matter, also called Total Solids (TS) is formed by organic and mineral matter (ash). In wheat straw, organic matter or Volatile Solids (VS) correspond to 39.6%TS of cellulose, 26.6% TS of hemicelluloses, 21% TS of lignin and soluble compounds (≈ 12% TS) in average (Monlau et *al.*, 2013; Sun, 2010) (see also Annex 1). During anaerobic digestion process, only organic matter is digested but lignin, hardly hydrolysable, remains. Moreover, water is conserved in the medium. In straws, water amount is low that is why Solid-State Anaerobic Digestion (SSAD) is better suited to straws fermentation.

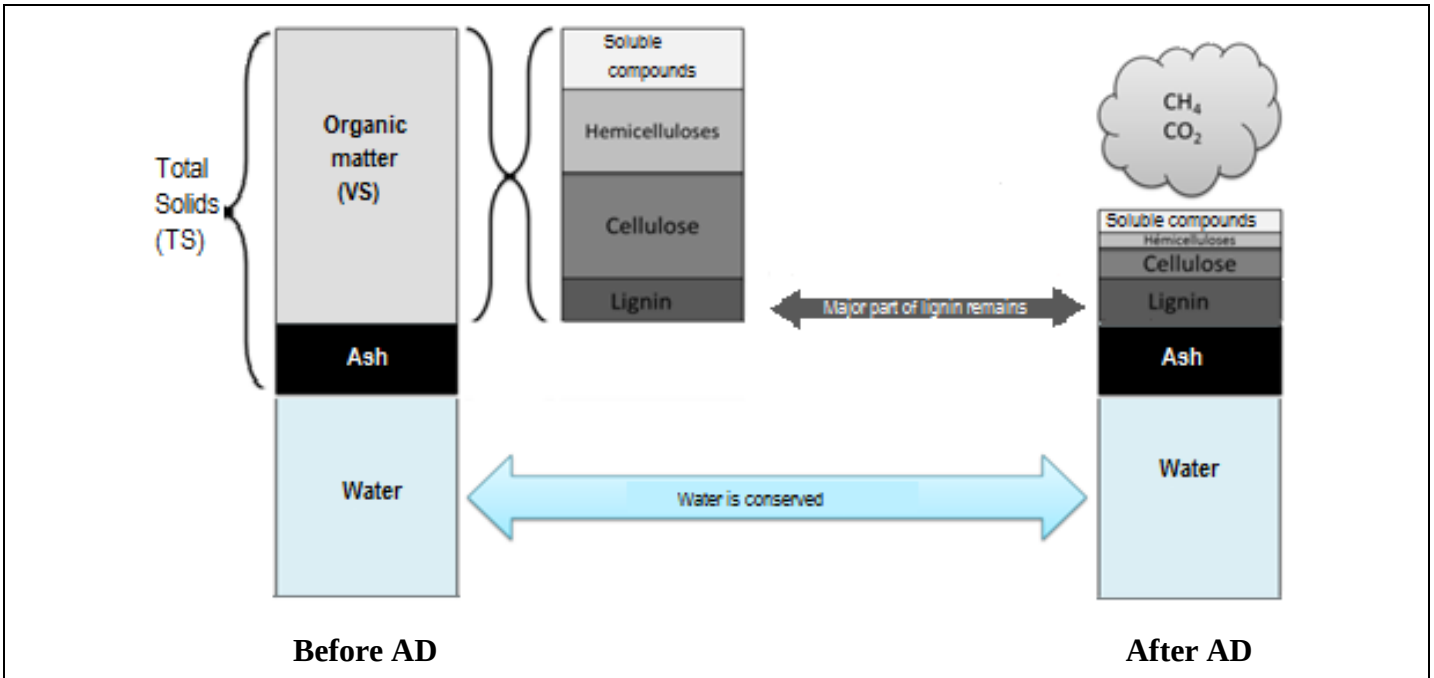


Figure 1-5. Schematic representation of lignocellulosic biomass composition before and after anaerobic digestion (AD).
(Adapted from Motte, 2013)

1.1.3 Solid-State Anaerobic Digestion: definition, advantages and limits

It is often considered that SSAD processes correspond to anaerobic digestion with more than 15% Total Solid (TS) even if the term semi-dry anaerobic digestion appears for TS content between 15 and 20% (Li et *al.*, 2011; J.-C. Motte et *al.*, 2013). The relatively recent interest of researchers for this process would explain the lack of a standardized term to designate this technology that can be qualified as dry, high-solid or also solid-phase and solid-state. Martin (2001) proposes the universal use of SSAD. No digestion can actually be 'dry' since lack of moisture limits bacteriological activity (Kusch et *al.*, 2011). In Europe, SSAD is particularly present (more than 60% of the capacity in substrate weight per year) (De Baere, 2000) although it represents only a small part of research works on anaerobic digestion (Motte, 2013) and that it is still uncommon in agricultural biogas plants (Kusch et *al.*, 2011).

Most plants operate under 30% TS content but it can reach 40% (Liew et *al.*, 2011). The highest is the TS content, the more mass diffusion limitation can be encountered with the possibility to inhibit hydrolysis step (Xu et *al.*, 2014). TS content is calculated at the beginning of the process but it decreases with organic matter degradation during anaerobic digestion. TS content determines the medium rheology, the dilution of the liquid phase or the moistening homogeneity (Figure 1-6). With very high TS content (>30%) methanogens *Archaea* selection occurs to keep most resistant species (to acidic pH, notably), there is also a shift in metabolism since major VFA produced become butyric acid instead of acetate (Motte, 2013; J.-C. Motte et al., 2013). The water rarefaction also leads to mass transfer limitation, notably gas-liquid transfer. Gas-liquid transfer are necessary for the functioning of the anaerobic digestion ecosystem to ensure proper exchanges between populations and avoid inhibitions (e. g. H_2 inhibition…) (Abbassi-Guendouz et *al.*, 2012).

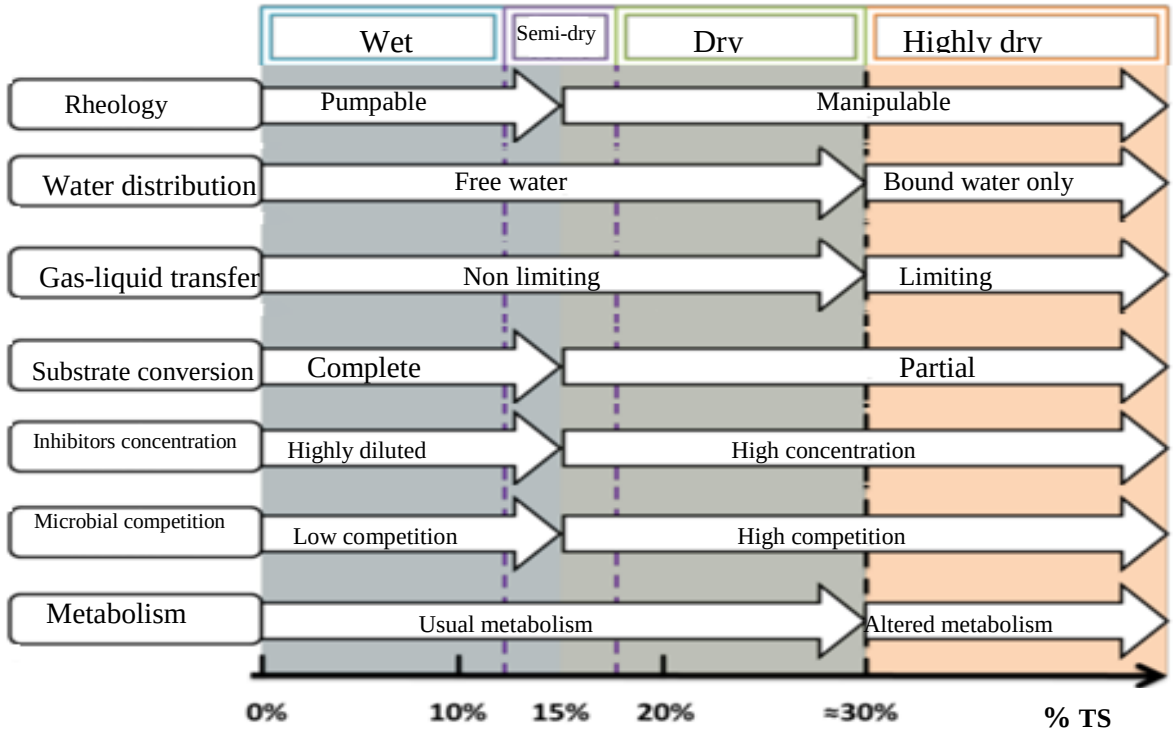


Figure 1-6. Effects of total solids content on anaerobic digestion.
(Adapted from Motte, 2013)

If biological process is the same in wet and dry processes, there are several particularities to operate solid-state anaerobic digestion. Reactors start-up is a particularly difficult step in SSAD (Jha et *al.*, 2011). SSAD **processes are less expensive (especially batch process) owing to smaller** and simpler reactors (few moving parts, lower energy requirement for heating) and digestate management is easier due to the absence of phase separation (Li et *al.*, 2011). Lignocellulosic substrates, like straw, have a tendency to float on the top of liquid reactors leading to operating difficulties and even to a lower methane production (Liao et *al.*, 1984). Moreover, the presence of inorganic or inert matter (sand, stones, plastics…) in wet process often leads to technical failures (corrosion…) and the substrate must be carefully prepared. Wet processes require complete mix reactors that lead to the occurrence of short-circuiting i. e. the output of digested matter before the planned duration for anaerobic digestion. Short-circuiting diminishes the biogas yield (Vandevivere et *al.*, 2003). However, several difficulties are encountered with SSAD owing to low moisture content, e. g. inhibitors concentrations or lack of homogeneity inside the reactor (Table 1-2).

Table 1-2: Interest of SSAD compared to wet AD processes.
(Karthikeyan and Visvanathan, 2013; Motte, 2013; Rapport et *al.*, 2008)

Criteria	Advantages	Disadvantages
Technical	• Few moving parts within the reactor which limit short circuiting and operational problems • Robust, no need to remove inert material and plastics since reactor not prone to abrasion from sand and grit	• Not appropriate for wet (TS <5%) waste streams
Biological	• Less VS loss in substrate preparation (no inert removal…) • Larger biomass amount • Less dispersion of inhibitors (less mixing and water)	• Low dilution of inhibitors with fresh water • Less contact between microorganisms and substrate (without inoculation loop)
Economic and Environmental	• Cheaper substrate preparation • Very small water usage • Smaller heat requirement (smaller volume) • Less wastewater and more compost formed as the final product	• Robust and expensive waste handling equipment required • Possible disminushing of theed substrate conversion rate

Under 30% TS content and with optimal SSAD (adequate Substrate/Inoculum ratio…), laboratory and full-scale reactors achieve biogas production per reactor volume comparable to those obtained in liquid-phase digestion. However, suboptimal conditions resulted in an inhomogeneous and incomplete degradation at farm-scale. Optimal conditions are difficult to be determined for full scale SSAD (Kusch et *al.*, 2011), notably because diverse parameters influence the reaction such as temperature and mixing.

1.1.4 Main parameters influencing anaerobic digestion

AD processes are classified by critical operating parameters and reactor design such as continuity (batch versus continuous), reactor design (plug-flow, complete-mix, and covered lagoons), operating temperature (psychrophilic, mesophilic and thermophilic), and solid content (wet versus dry) (Li et *al.*, 2011).

1.1.4.1 Temperature

Three ranges of temperatures are used to perform anaerobic digestion: psychrophiles, mesophiles and thermophiles (Figure 1-7). Each of this temperature range corresponds to species most able to live at these temperatures but there is not a clear boundary between classic groups of psychrophilic, mesophilic and thermophilic microorganisms (Lettinga et *al.*, 2001). At industrial scale mesophilic (optimum ≈ 37°C) and thermophilic (optimum ≈ 55°C) temperatures are generally employed (Jha et *al.*, 2011). Thermophilic conditions helped the successful start-up of dry anaerobic digesters with food wastes without too much VFA accumulation (Lu et *al.*, 2007) whereas with corn stover, it have led to increased VFA accumulation (Shi et *al.*, 2013). Thermophilic conditions act on hydrolytic enzymes activities (Lu et *al.*, 2007) and hydrolytic microorganisms population abundance. Thermophilic systems against mesophilic are reported to be more rapid and to sometimes improve methane yield but they are also less stable in performance and sensitive to small temperature changes and to inhibitors. Moreover, they require increased energy for maintaining the reactor at the higher temperature that is why mesophilic systems are the most used (Jha et *al.*, 2011). Reactors can be heated with a part of the biogas. Under SSAD conditions, reactors are smaller and less energy is required to heat them thereby it is easiest to have positive earnings using thermophilic conditions than with liquid AD. Small water amount in SSAD generally induces more thermal heterogeneity (Motte, 2013).

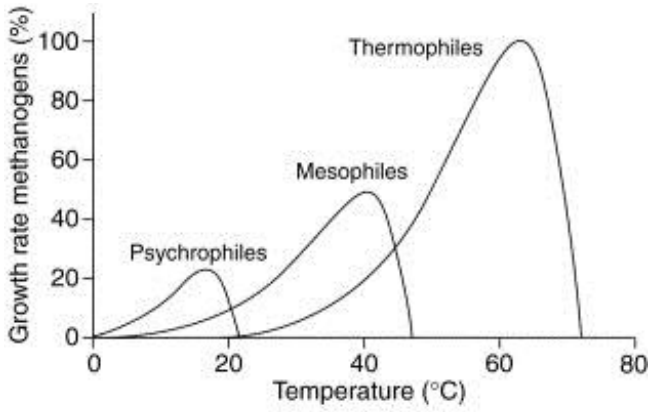


Figure 1-7. Relative growth rates of psychrophilic, mesophilic and thermophilic methanogens.
(Lettinga et *al.*, 2001)

1.1.4.2 Mixing

Mixing allows an enhance contact between enzymes, substrates (and nutrients) and microorganisms. It also promotes heat transfer and release of produced gas from the digester contents. This release is useful to avoid

sedimentation (Jha et *al.*, 2011). Moreover, gas accumulation (H_2, H_2S…) can affect the overall process performance (Karthikeyan and Visvanathan, 2013). Inactive zone formations (more frequent in SSAD), affecting bioconversion can impact biogas yield (Karthikeyan and Visvanathan, 2013; Kusch et *al.*, 2011). However, excessive mixing can reduce the gas production and even lead to failure with high organic loading rate (Gómez et *al.*, 2006; Vavilin and Angelidaki, 2005). A destructuration of microbial compartments may explain this fact. Microorganisms would be exposed to inhibitors which they were previously protected due to the structuration of communities (Martin, 2001; Vavilin and Angelidaki, 2005). Limited mixing represents energy saving. Mixing is carried out by several ways: mechanical agitation, biogas recirculation in the substrate, or even, for SSAD, by leachate recycle (Jha et *al.*, 2011). Mixing during start-up phase is not beneficial and increases pH drop (Karim et *al.*, 2005). In SSAD process, mixing is difficult and comparatively expensive (Jha et *al.*, 2011). Furthermore, in dry conditions mixing with biogas recirculation is possibly ineffective (Karim et *al.*, 2005). Leachate recycle can be used to favor homogeneity (temperature, microorganisms, nutrients…). This action is limited since preferential pathways often appear (Motte, 2013) but help to maximize methane production per reactor volume (Kusch et *al.*, 2011).

1.1.4.3 pH and alkalinity

Even if optimum pH range for anaerobic digestion is always neutral, slight variations can be found in literature (Angelidaki and Sanders, 2004; Jha et *al.*, 2011). Optimum pH for methanogens is between 7 and 8 (Angelidaki and Sanders, 2004) and for hydrolysis and acidogenesis is between 5.5 and 6.5 (Jha et *al.*, 2011). Anaerobic digestion can occur between 6 and 8.3 (Angelidaki and Sanders, 2004). pH influences the toxicity of inhibitors modifying the proportion of ionized and non-ionized forms. Thereby, ammonia is toxic when the pH is above 7, while VFAs and hydrogen sulfide are more toxic below pH 7; even if microorganisms can sometimes acclimate (Jha et *al.*, 2011). Alkali addition to approach neutral pH can reduce the time to start a SSAD reactor (until 250 days in worst cases) (Karthikeyan and Visvanathan, 2013).

Alkalinity is related to the buffer capacity of the medium. A sufficient alkalinity is required during anaerobic digestion, it can notably help to face high VFA concentrations without detrimental pH decrease. Alkalinity in stable reactor is frequently between 2 and 4 g $CaCO_3$/L (APHA, 1998). This parameter is often measured thanks to titrimetric methods since they are cost-effective, rapid and simple. However, it exist lot of measurement techniques with different accuracy. Among them, Ripley et *al.* (1986) technique provides a good compromise between ease of execution and accuracy. Suspended solids like struvite or humic substances (especially present in manure) can alter the measurement. A filtration to 0.45 µm is thus recommended (Scherer, 2007) that is sometimes replaced by a centrifugation step (Ripley et *al.*, 1986; E Voß et *al.*, 2009).

1.1.4.4 Carbon/Nitrogen (C/N) ratio

It is well known that anaerobic digestion substrate must have a C/N ratio adapted to microorganisms requirements. Optimal ratio is between 20 and 35, lower ratios lead to potential inhibition with ammonia whereas higher ratios may lead to nitrogen limitations (Sialve et *al.*, 2009). Certain reactor designs (two-stage) would tolerate lower ratios. Crop residues have generally a C/N ratio higher than required, e. g. up to 161 for wheat straw (McKendry, 2002b). Co-digestion with a substrate rich in nitrogen (sludge, manure…) is a good way to adjust C/N ratio and eventually to improve methane production (Ward et *al.*, 2008). Co-digestion promotes digestion kinetics. It can also be used to provide buffer capacity, to reduce external nutrient supply or even to improve digestate quality (Karthikeyan and Visvanathan, 2013).

1.1.4.5 Nutrient supplementation

Diverse macro- and micro- nutrients are required for optimal work of anaerobic microorganisms. Micro elements like iron, cobalt, nickel, etc., can be co-enzymes or co-factors for the biosynthesis of CH_4 or be involved in the growth of anaerobic microorganisms. Poor nutrient content of anaerobic digestion medium leads to low CH_4 yield and process instability. Moreover, it also affects the digestate quality. Macro- and micro-nutrients addition is required (see Chapter 2, 2.1.5, for medium composition), unless it can be documented that they are present in inoculum or substrate. Co-substrate can sometimes provide missing nutrients. Limited studies are available concerning nutrients in SSAD process (Angelidaki et *al.*, 2009; Karthikeyan and Visvanathan, 2013).

1.1.4.6 Inhibitors

Diverse inhibitors commonly present in biomass are encountered in anaerobic digesters: ammonia, sulfide, light metal ions (Na, K…), heavy metals (cadmium, nickel…) and organics (phenols, benzenes…). The inhibition concentration ranges vary widely for specific toxiccompounds since a lot of parameters can influence the toxicity (anaerobic inocula, waste composition, and experimental conditions). Microorganisms are sometimes able to adapt to inhibitory substances, especially with low concentrations. Certain technics exist also to remove or counteract toxicants, e. g. activated carbon against sulfide or Na^+, Ca^{2+}, and Mg^{2+} addition to fight ammonia inhibition (antagonists) (Chen et *al.*, 2008).

1.1.4.7 Particle size of the substrate

As hydrolysis is often the limiting step, a reduction of particle size generally improves methane production rate. Particle size is considered as a major parameters in kinetic studies but would not improve the total amount of produced methane (Angelidaki et *al.*, 2009). However, some studies report that increased methane amount can be obtained thanks to particle size diminishing and in particular for lignocellulosic biomass but it seems to concern very small particle size (< 1 mm) that are not found in industrial biogas plants (Jędrczak and Królik, 2007; Palmowski and Muller, 2000). Moreover, particles size reduction with

SSAD increases the risk of acidification because of rapid and high acids production that are not enough diluted by the little available water (Motte et *al.*, 2014b).

1.1.4.8 Inoculum

Inoculum must contain all microorganisms required for anaerobic digestion. The use of digestate (solid and/or liquid) from a previous anaerobic digestion is common to inoculate the substrate. When substrate is similar to the one of the previous digestion, the inoculum is already adapted and anaerobic digestion can start easily. Digested sewage sludge or manure are also currently used as inoculum (Karthikeyan and Visvanathan, 2013). Inoculation step allows the contact between microorganisms and substrate, it is particularly important in SSAD processes since microorganisms are little transported by water or mixing (Motte, 2013). High inoculation ratios are generally used in SSAD to maximize the contact with substrate but also to avoid acidification. An important acidification period due to imbalance between acid production and consumption leads to the death of methanogens. To avoid this situation and decrease digestion duration, a sufficient amount of methanogens must be provided by the inoculum. However, the higher the inoculum amount, the lower the substrate amount and thus the lower methane production per reactor (Ahring et *al.*, 1995). Amount of required inoculum strongly depends on specific substrate characteristics and may vary within a wide range (ensiled maize or grass: around 70% w/w based on TS; horse dung with straw: 10 to 20% w/w TS; cattle dung: 0%) (Kusch et *al.*, 2011). Some quite low substrate/inoculum ratios are employed in plants and this ratio must be optimized. In contrast, very high ratios were sometimes used for research convenience (2%) resulting in a long start-up phase (month) not adapted to industrial requirements. With SSAD, it was shown that substrate/inoculum (S/I) influences principally the start-up phase whereas the growing phase depends probably of water compartmentation (TS content and particle size) (J. C. Motte et *al.*, 2013). To finish, inoculum efficiency is function of its microorganism communities and of substrate used but other abiotic parameters can also have an important influence on anaerobic digestion such as nutrient content or buffer capacity (Shi et *al.*, 2014b). Besides, optimum C/N ratios in reactors take into account the part coming from inoculum (often rich in N).

1.1.5 Anaerobic digestion processes

1.1.5.1 Biochemical Methane potential (BMP)

1.1.5.1.1 Definition and measurement

Biochemical Methane Potential (BMP) measurement is central for anaerobic digestion characterization of a substrate. The BMP of a substrate is the maximum methane quantity produced by degradation of this substrate. It is expressed in methane volume per substrate mass unit. As gas volume is a function of temperature and pressure, gas volumes are expressed in NormoLiters i. e. volume in Standard conditions for temperature and pressure (1 atm, 0°C). BMP of the planned feedstock are measured before biogas plants construction to estimate their profitability and size since it represents the aptitude of a substrate to generate

energy. In operating plants, BMP can also help to predict the behavior of a new input or to optimize the composition of substrate mixtures. If several standards and recommendations exist for the BMP measurement (BMP test), there is no standard protocol. With simple substrate, BMP tests precision can vary from 20% between laboratories (Bachmann et *al.*, 2011; Raposo et *al.*, 2011). BMP tests that consist in anaerobic digestion in optimized conditions, must be carried out in triplicates. In addition, even if some authors found no influence of inoculum origin on quite degradable substrate (Raposo et *al.*, 2011), with toxic substrate such as phenol influence of inoculum source was clear and a correlation was found between extent of degradation and specific methanogens activity (maximal slope of methane production curve) (Moreno-Andrade and Buitron, 1995). Therefore, if a comparison between substrates is required, BMP tests must be launched at the same time to ensure same conditions, especially to have the same inoculum.

BMP is defined as a maximum obtainable methane yield that is why micro (Fe, Mn…) and macroelements (N, Ca…) are generally added before the anaerobic digestion to ensure microorganisms needs. To keep neutral pH, a buffer solution must also be used. BMP tests are realized in liquid medium. Agitation must be providing during the reaction. Temperature is fixed at whether thermophilic or mesophilic conditions depending on the goal of the BMP determination but mesophilic conditions are more widespread. Various precautions should be followed concerning the inoculum for BMP tests. This inoculum comes from anaerobic reactors and must be kept warm (mesophilic or thermophilic temperatures). Inoculum is better preserved in absence of oxygen and with regular organic matter input to 'feed' microorganisms. Activity of the inoculum can be followed using an entirely biodegradable substrate such as ethanol. This high biodegradability confers to ethanol a little variable BMP equals to 730 mL CH_4 per g. Thus, known amount of ethanol can serve as control substrate during the BMP tests to follow inoculum efficiency: if measured BMP is too far from known BMP, it means that inoculum is no more active. Endogenous methane production of the inoculum must also be measured, it corresponds to methane production of the inoculum alone without substrate and in the same conditions as those for BMP tests. This methane production can be due to residual substrate in the inoculum or also to the consumption of death bacteria. Endogenous production is removed from total methane production measured during BMP test in batch with substrate in order to obtain only methane production due to the substrate. Thus, endogenous production must be largely inferior to substrate methane production to allow detection of little methane production variations between samples. Finally, sufficient amount of inoculum (I) must be provided regarding to the substrate (S) quantity. A high amount of inoculum allows facing potential inhibitors issued from substrate degradation. Without inhibition mechanisms, S/I (VS basis) between 1 and 6 had no influence on BMP values of cellulose (less than 10%) (Bachmann et *al.*, 2011). However, with kitchen wastes, it was shown that S/I is particularly impacting when added alkalinity is low since acidification can occur (Neves et *al.*, 2004).

1.1.5.1.2 Experimental and theoretical BMP values: example of wheat straw

BMP is a characteristic value of the substrate that reflects its biodegradability. BMP values range between 0 to 1000 m^3 CH_4/t VS (Raposo et *al.*, 2012). Fats have a particularly high BMP value whereas ligneous

wastes have a lower BMP (≈250 m^3 CH_4/t VS). BMP are function of the composition and of the structure of the substrate. BMP of a given substrate, e. g. wheat straw, can vary in a wide range (Figure 1-8) even if experimental conditions for the BMP tests are identical (same inoculum, etc.). This is due to the variable biochemical composition for a same species reflecting growth stage, availability of nutrient during the plant culture, etc. Moreover, if the feedstock is not well stored, degradation can occur before the entrance in anaerobic digestion reactor leading to a lower methane production. For wheat straw, average BMP value obtained with mesophilic temperature reaches 225 NmL/g TS taking into account thirteen experiments (Figure 1-8). The average standard deviation corresponds to 23% (Annex 1). With thermophilic temperature, BMP of wheat straw is 265 NmL/g TS in average (Figure 1-8) but only three studies were taken into account that would explain the lower variability in results and the highest value obtained in thermophilic conditions (Standard-deviations, minimum and maximum values in Annex 1). However with thermophilic SSAD, it was reported a better efficiency for wheat straw anaerobic digestion (methane production-rate and yield) due to a higher hydrolysis rate (Pohl et *al.*, 2012).

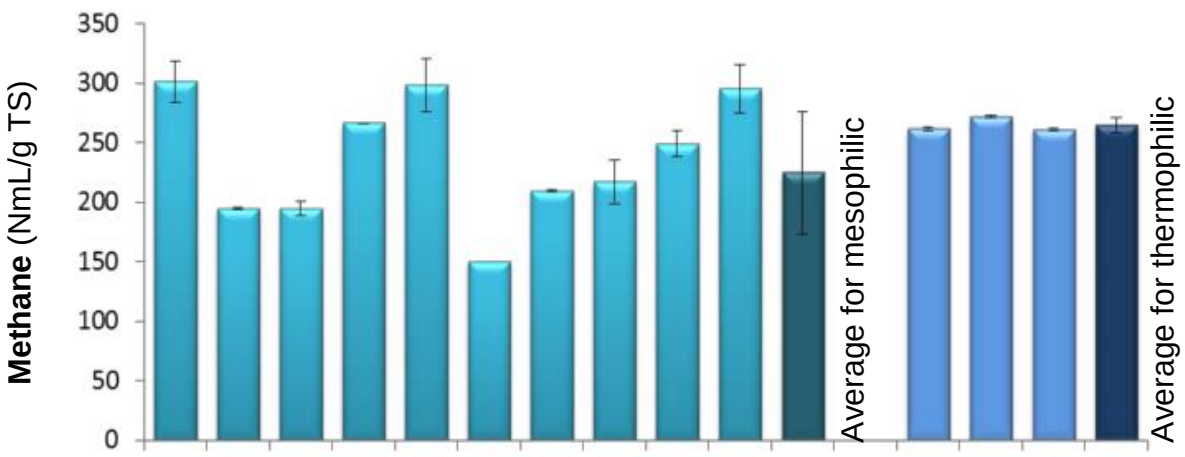


Figure 1-8.Wheat Straw Biochemical Methane Potential from literature.
(Motte, 2013)

BMP corresponds to the value reached at the plateau phase of methane production (called "stationary" in Figure 1-12A). It can take few days (e. g. for ethanol, highly biodegradable) or months (e. g. wheat straw, little biodegradable) to obtain this value. For entirely biodegradable substrate (glucose solution, etc.), the BMP can be determined by a Chemical Oxygen Demand (COD) measurement. It is admitted that 1 g of COD produces about 350 mL of CH_4 (Lesteur et *al.*, 2010). COD measurement are generally realized on liquid substrate (Buffière et *al.*, 2009). The Buswell formula, using a stoichiometric equation based on the chemical composition of the substrate ($C_nH_aO_bN_c$), can also be useful to calculate the theoretical amount of methane produced (Lesteur et *al.*, 2010). The Buswell formula is expressed as follows:

$C_nH_aO_bN_c + \left(n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4}\right)H_2O \rightarrow \left(\frac{n}{2} - \frac{a}{8} - \frac{b}{4} + \frac{3c}{8}\right)CH_4 + \left(\frac{n}{2} - \frac{a}{8} - \frac{b}{4} + \frac{3c}{8}\right)CO_2 + cNH_3$. However, chemical composition includes all organic matter even non-biodegradable ones (such as lignin), that is why

Buswell Formula is not adapted to determine the BMP of not entirely biodegradable substrate. Moreover, this formula does not take into account the part of substrate not converted to methane because used for microorganisms growth (Lesteur et *al.*, 2010).

With Buswell formula, calculated BMP for wheat straw is 466 NmL/g TS (Annex 1. *Characterization of wheat straws in literature*.). Comparing to the experimental value (225 NmL/g TS), it means that wheat straw biodegradability is inferior to 50% (Motte, 2013). This is notably due to the presence of lignin that cannot (or very poorly) be converted into methane. Thus, wheat straw requires high digestion time, it is estimated that only 49% of the methane potential is obtained in 26 days and 61% in 42 days in industrial reactor (Kusch et *al.*, 2011).

As BMP tests produce methane in optimum conditions, studies in conditions closest to industrial scale must also be carried out to identify possible failure (deficiency, inhibitions etc.). In addition, BMP tests are realized in liquid medium and would not be enough representative of SSAD. In SSAD, inhibitions are most current, notably acidification, because there is little water to dilute inhibitors. In 2001, Federal Government of Germany recognized the GB_{21} tests for solid wastes, it consists in a digestion of 50 g TS with 50 mL inoculum and 300 mL water during 21 days (Ponsá et *al.*, 2008). To our knowledge, the relationship between methane production from GB_{21} and BMP tests is unknown. For these reasons, studies at reactor scale are required to better predict behavior in full scale plants.

1.1.5.2 SSAD processes

Several reactor configurations exist, one of the most discriminant aspects concerning existing technologies is the functioning mode: continuous or discontinuous. Among the several reactor designs existing for SSAD, no one is more attractive than others and it must be chosen taking into account substrate characteristics (Karthikeyan and Visvanathan, 2013).

1.1.5.2.1 Batches

Discontinuous processes also called batches are characterized by a closed reactor in which anaerobic digestion proceeds during a pre-defined period. There is no in and out flux for solid matter during this period. When this period is over, digestate is removed and fresh substrate mixed with inoculate is loaded to begin a new reaction. Biogas supply is not continuous since digestion and methane production start again with each filling of the reactor (Figure 1-12C). To avoid excessive biogas fluctuations, at least three reactors should run off-set (alternative loading and unloading) (Kusch et *al.*, 2011).

Leachate recycle is generally used to favor anaerobic digestion (temperature homogenization, substrate moisture, microorganisms transportation) (Brummeler et *al.*, 1992; Buffière et *al.*, 2009). Batch technologies can differed in their management of leachate (Figure 1-9). Batch systems such as tunnel anaerobic digestion (Biocel® technology) have a chamber to collect leachates that are then sprayed on top of the wastes that are being digested (Figure 1-9A). In France, tunnel anaerobic digestion is comercialized by Naskeo Methajade.

Leachates can also be recycled between new and mature reactors in the Sequential Batch Anaerobic Composting technology (SEBAC) (Figure 1-9B). This allows a better process stability due to a lower risk of acidification. In older reactor, leachates have less acids than in new reactor since in older reactor time was sufficient for acids consumption (Chavez-Vazquez and Bagley, 2002).

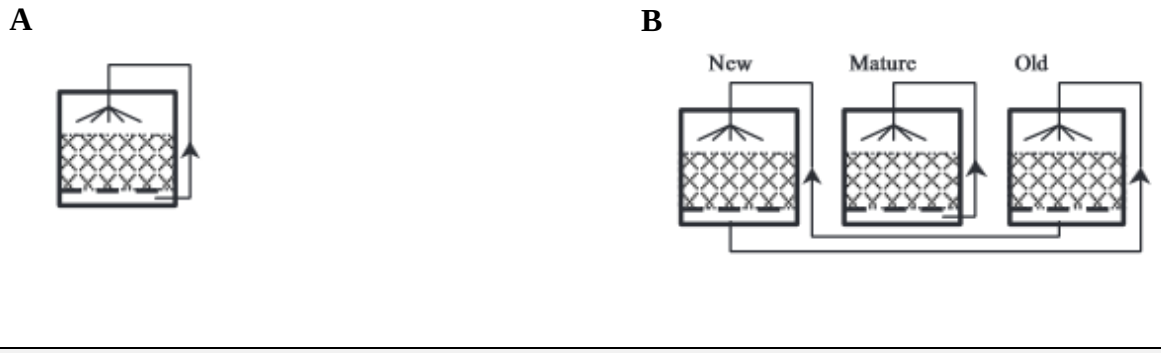


Figure 1-9. Examples of leachate recycle patterns in batch systems.
A) Single stage. B) Sequential batch. (Lissens et *al.*, 2001)

1.1.5.2.2 SSAD continuous processes

SSAD continuous process reactors were notably developed for treatment of Organic Fractions of Municipal Solid Waste (OFMSW). Substrate addition and digestate removing occur at regular intervals. SSAD continuous reactors are based on plug flow, materials added on one end of the digester push older materials toward the opposite end. Reactors are partially mixed to inoculate the incoming feedstock and limit localized acidification. Three main reactor designs are currently employed (Figure 1-10): Valorga, Dranco and Kompogas (Rapport et *al.*, 2008). Those designs are distinguished by their mixing systems (Moletta, 2008). Kompogas process (Figure 1-10A) is a horizontal steel tank with slowly rotating axial mixers that contribute to push the material to the outlet, to avoid decantation and favor degassing (Li et *al.*, 2011). Dranco technology (Figure 1-10B) corresponds to vertical silo design with a conical bottom auger discharge. It is mixed by digestate recycle outside the tank (up to 6/1 parts of recycled digestate/fresh OFMSW). Finally,Valorga reactors (Figure 1-10C) are agitated thanks to biogas injection into the waste on the bottom of the reactor. They are vertical steel tanks with a central baffle that pushes the material from the inlet to the outlet port. Principal limitation of this process is that gas nozzles can clog.

Dranco and Kompogas systems operate at thermophilic temperature. Kompogas is the most widespread design probably because, the retention time is short (15-20 d) and thus more OFMSW can be digested during a given period.

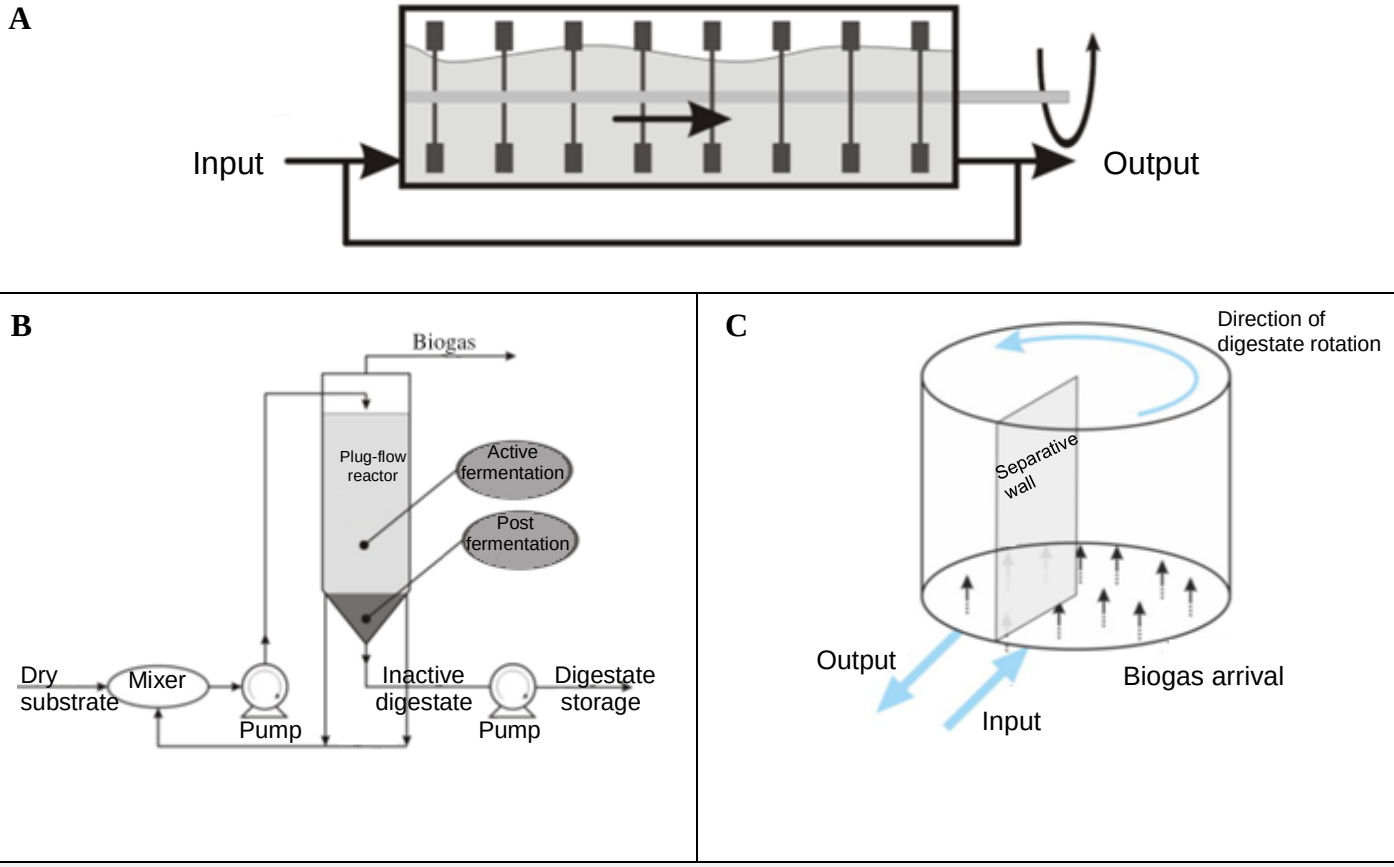


Figure 1-10. Three main continuous reactor designs.
A) Kompogas process with axial mixer to move digestate. B) Dranco process, use of gravity for digestate progression. C) Valorga process, agitation with biogas injection. (Adapted from Motte, 2013)

Finally, continuous SSAD processes can also be realized with two reactors (technology Arkometha). Two-step processes consist to separate the hydrolysis/acidogenesis steps from acetogenesis and methanogenesis steps thanks to two reactors in series (even if acetogenesis sometimes occurred with hydrolysis/acidogenesis steps) (Figure 1-11). This configuration allows a best management of the biological reaction as operating conditions are different in each reactor (e.g. more acidic conditions in hydrolysis reactor or possibility to work in aerobic conditions for hydrolysis…). This can allow a more efficient hydrolysis (often limiting step) and a better process stability (lower risk of acidification).This process allow the recuperation of hydrogen produce in the first reactor. One-step processes are the most curent in Europe (≈ 90% of anaerobic digestion capacity) (De Baere, 2000). Two-step processes allow higher loading rates, improved stability and flexibility but the added complexity and presumed expense of building have negated the possible yield and rate enhancements (Rapport et *al.*, 2008).

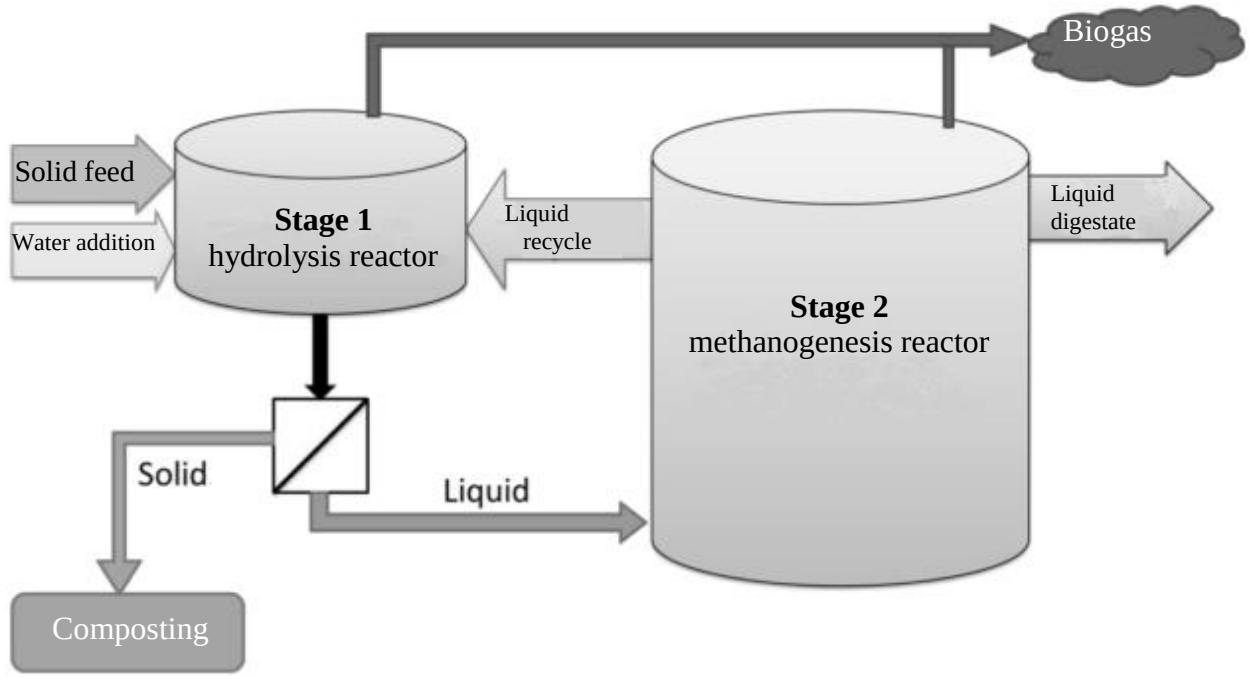


Figure 1-11. General concept of two-stage reactors.
(Motte, 2013; Adapted from Rapport et *al.*, 2008)

1.1.5.2.3 Batch VS continuous

Batch systems require less capital cost and are relatively simpler notably because heavy process equipment are not required to mix dry viscous material during the reaction (Li et *al.*, 2011). Continuous mode is particularly adapted to substrates that are regurlarly produced in high amount or that are difficult to store (highly putrescible…) (Buffière et *al.*, 2009). However, charging/discharging cycles in batch processes require agricultural machinery and the regular opening of the digesters (odors and noise) that is why they are most adapted for sparsely populated areas (Motte, 2013). Batch processing prevails in agricultural solid-state anaerobic digestion. Crop residues-containing biomass (solid dung, green cut, wheat straw…) is especially interesting in batches because it can prevent problems of compaction that impede a proper leachate recycle (Kusch et *al.*, 2011).

Performances of optimised continuous SSAD plants are comparable to wet digestion with similar methane yield and volumetric productivity (methane per reactor volume unit per day) (Chen et *al.*, 2014). Maintenance and technical equipment requirements are more important (pump for high viscosity fluid, additon of micronutrients…).

Lower gas yields are sometimes encountered in dry discontinuous reactors than in continuous. Notwithstanding, explotation of dry batches is robust, failure rarely occurs and less control measures are required. Thus, there is no extensive need of operators training (Karthikeyan and Visvanathan, 2013; Kusch et *al.*, 2011).

Methane production in batch can be linked to bacterial growth: lag phase, acceleration phase, exponential phase, stationnary phase. Thus the kinetic of production is not constant and there is a peak of productivity during “exponential phase” (Figure 1-12B). Then, slow methane production continues during quite a long time. The duration of anaerobic digestion in plants is generally issued from a sufficient substrate degradation for a satisfiing methane production. Methane production in continuous processes is more regular. Their functionning is sometimes comparable to a succession of batch reactors owing to the plug-flow and the low mixing (Figure 1-12D). Methane production is variable depending on the reactor area and thus on the stage of completion of the degradation. Addition of all local methane production allows a quite stable production. Studies in batch reactors can be usefull to collect data on local functionning in continuous processes (Motte, 2013).

1.2 Pretreatments for anaerobic digestion

Pretreatments can be applied to increase methane yield or anaerobic digestion rate and sometimes to circumvent harmful substrate specificities (e. g. sanitation of animal by-products…). To be economically viable, pretreatment cost must be sufficiently inferior to its economical gain (represented by methane production improvements). Without pretreatment, energy input/output for biogas plant is estimated to 35% for straw considering a distance of 10 km between field and biogas plant (Berglund and Börjesson, 2006). Thus, a pretreatment step must increase this ratio. For lignocellulosic biomass, hydrolysis (limiting step) must be favored and in this goal enzymatic accessibility must be improved (Bertrand et *al.*, 2006). To do that, the major aim of lignocellulosic biomass (LB) pretreatments is delignification (Carrere et *al.*, 2014). However, other beneficial effects occur (listed in Figure 1-13) such as specific surface area increase that will also favor enzymatic accessibility.

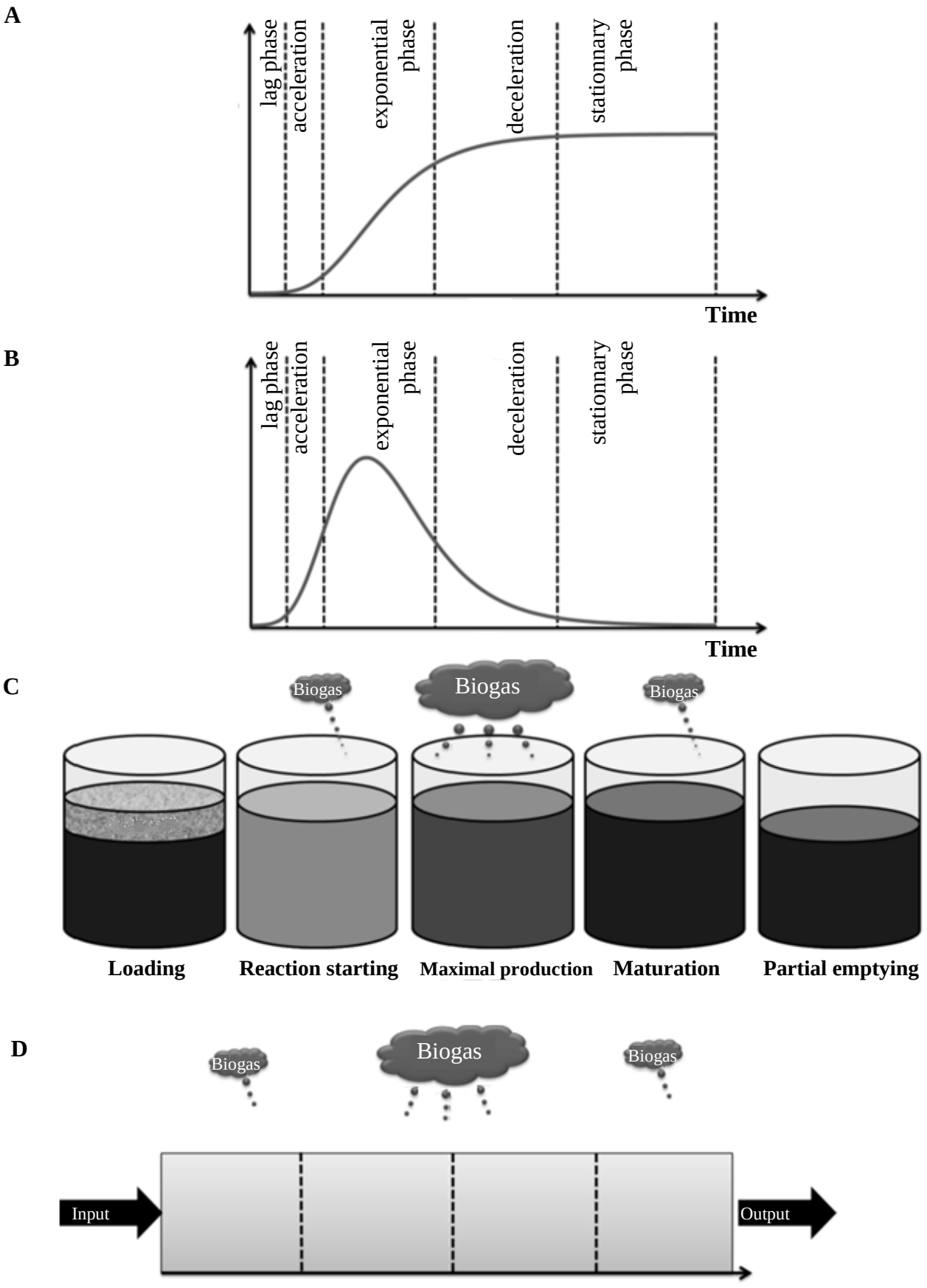


Figure 1-12. Evolution of biogas production with reaction duration.

A) Cumulated biogas production in function of time. B) Biogas production rate in function of time. C) Variable biogas production in a single batch reactor and necessity of multiple batch reactors to limit fluctuations of biogas supply. D) Similarity of continuous plug-flow reactor with multiple batch reactors. (Adapted from Motte, 2013)

Lignocellulosic biomass pretreatments can be divided in different categories: mechanical or physical, chemical, thermal, thermo-chemical and biological (Monlau et *al.*, 2013). Mechanical pretreatments are designed to physically reduce the size of LB particles. Among them, the simplest technique is milling but other methods such as radiations or microwaves can be used. Thermal pretreatments are based on the use of a solvent (water, CO_2, etc.) at high temperature and sometimes in combination with a high pressure. Chemical pretreatments require either acids or strong bases, either oxidants, either organic or ionic solvents. Organic solvents appear to be particularly effective in promoting the digestibility of substrates. They are heated to favor the reaction (organosolv pretreatment). Talebnia et *al.* (2010) schematized main preatreatment methods studied on wheat straw and their possible qualitative influence on substrate (Figure 1-13). Pretreatment classification can be slightly different among authors (e. g. in Figure 1-13, physico-chemical instead of thermo-chemical).

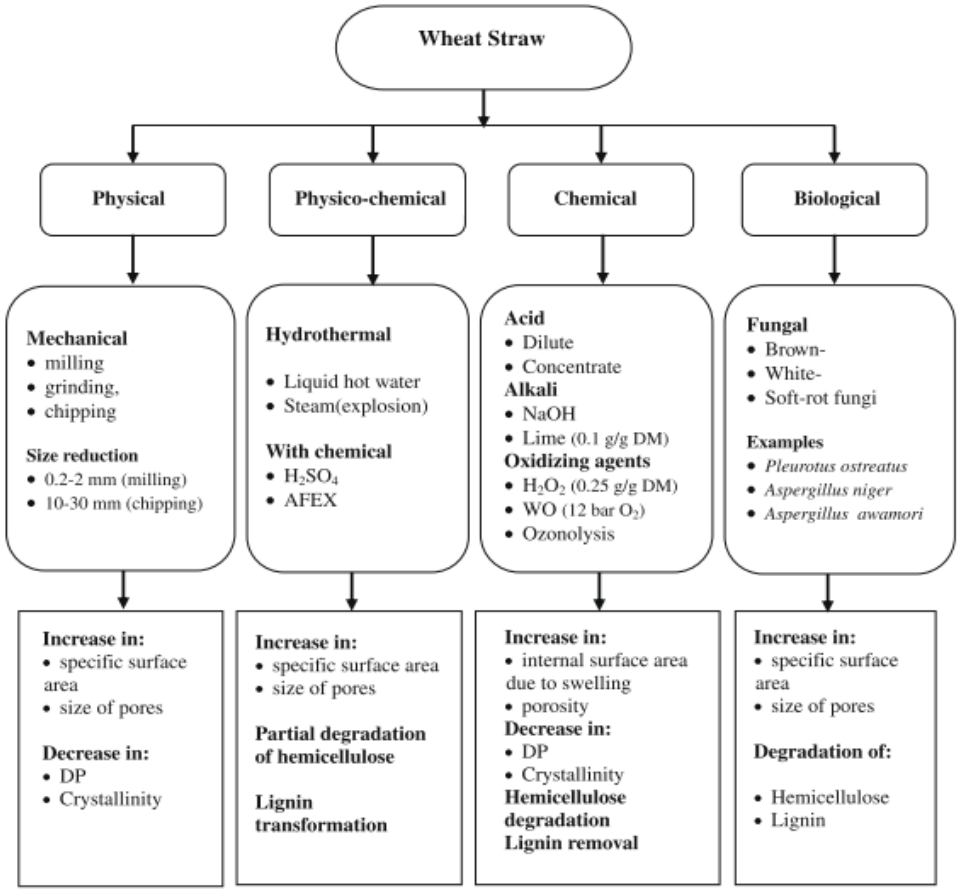


Figure 1-13. Most common pretreatment methods used on wheat straw and their possible effects.
(AFEX: Ammonia Fiber EXplosion DP: degree of polymerization; WO: Wet Oxydation) (Talebnia et *al.*, 2010)

1.2.1 Non-biological pretreatments

Chang et *al.* (1998) summarized digestibility improvements after wheat straw pretreatments (Table 1-3). Studied digestibilities were in majority evaluated with glucans hydrolysis. Digestibility improvements after non-biological pretreatments vary between 0% and 56% more compared to untreated straw (Table 1-3).

Table 1-3. Impact of pretreatment on wheat straw digestibility.

N. A.: Not Available. EDTA: Ethylenediaminetetraacetic acid. EDA: Ethylenediamine. DMSO: Dimethylsulfoxide.

[a]glucan conversion. [b]*in vitro* dry matter digestibility. [c]*in vivo* dry matter digestibility. [d]*in vivo* organic matter digestibility. (Adapted from Chang et *al*., 1998)

	Pretreatment	Temperature (°C)	Time	Water loading (mL/g TS)	Pretreatment agent loading or other conditions (g/g TS)	Particle size	Additional percentage of digestibility compared to untreated substrate (%)
PHYSICAL	Ball milling	–	4h	–	Rotation speed = 52 rpm	-10 mesh	**18.5**[a]
	Fitz milling	–	N. A.	–		-10 mesh	**3.5**[a]
	Roller milling	–	0.5h	–		-10 mesh	**9.5**[a]
	Extrusion	N. A.	N. A.	–		-10 mesh	**0.3**[a]
	γ-Ray irradiation	–	N. A.	–	Radiation dosage = 5 X 10^7 rad	-10 mesh	**13.5**[a]
	Steam explosion	230	1 min	–		5 cm	**35**[a]
CHEMICAL	NaOH	N. A.	N. A.	0.3	0.01-0.15	0.3175 cm	**48**[b]
	NaOH	129	2h	10	0.1	- 10 mesh	**56**[a]
	$Ca(OH)_2$	Ambient	72h	N. A.	1%	N. A.	**26**[c]
	$Ca(OH)_2$+NaOH	Ambient	24h	11	0.09	3 cm	**8**[d]
	$Ca(OH)_2$	Ambient	120h	1.5	4%+1%	2.54 cm	**35**[b]
	NH_4OH	25°C for 8h + 55°C for 72h		N. A.	25%	2.5-7.5 cm	**32**[a]
	EDTA	25°C for 8h + 55°C for 72h		N. A.	5% (v/w)	2.5-7.5 cm	**3**[a]
	H_2SO_4	98	1h	16	0.73	-10 mesh	**19**[a]
	Peracetic acid	100	0.5h		10 mL/g dry biomass	- 10 mesh	**49**[a]
	Ethylene glycol + HCl	129	1h	–	Ethylene glycol = 12.5 mL/g dry biomass, HC1 = 0.27 mL/g dry biomass	- 10 mesh	**49**[a]
	DMSO	25°C for 8h + 55°C for 72h		N. A.	5% (v/w)	2.5-7.5 cm	**5**[a]
	EDA	25°C for 8h + 55°C for 72h		N. A.	28% (v/w)	2.5-7.5 cm	**49**[a]
	Na_2SO_3	129	2h	6	0.96	- 10 mesh	**32**[a]
	NaOCl	129	2h	9.5	0.5	- 10 mesh	**50**[a]
	Butanol	175	1.5h	5	5 mL/g dry biomass	- 10 mesh	**5**[a]
	Multiple Fitz milling + NaOH	129	2h	10	0.1	- 10 mesh	**43**[a]

Pretreatments are often evaluated for their effect on hydrolysis (sometimes only on cellulose), it is not sufficient to evaluate their efficiency on AD. With 4% ammonium (w/v) pretreated hay, no methane production increase was observed even if hydrolysis rate was significantly enhanced. With wheat straw, steam explosion at 180°C for 25 min increased methane production by 31% (Monlau et *al.*, 2013) whereas no improvement was observed with a lower temperature (Theuretzbacher et *al.*, 2015). Fine grinding (0.4 mm) led to a 53% enhancement of the energy yield from wheat straw (Sharma et *al.*, 1988). Particle size reduction can improve both methane production yield and rate. On wheat straw, it was reported a 43% BMP increase with 10% NaOH at 40°C for 24 h and 67% with 10% NaOH at 100°C for 30 min (Carrere et *al.*, 2014).

Pretreatments can incur important additional costs for processes. These costs can be due to high energy consumption, chemicals, and eventually, expensive anti-corrosion reactors to resist harsh reaction conditions (Zhao et *al.*, 2014b; Zheng et *al.*, 2009). Additional production represent additional profits that must be superior to additional costs generated by the pretreatment. The evaluation of these costs is rare because it necessitates full-scale experimentations often expensive and complex. Moreover, pretreatments can reduce the environmental friendly advantages of anaerobic digestion by using chemicals and/or generating wastes (Mosier et *al.*, 2005). Some pretreatments (thermal or acid processes…) lead to the release of inhibitory by-products such as 5-HMF, furfural or phenols generated by the degradation of cellulose, hemicelluloses and lignin, respectively. Although there is a debate about their biodegradation during anaerobic digestion (Monlau et *al.*, 2014), a detoxification step is sometimes proposed (López-Abelairas et *al.*, 2013). Finally, sophisticated expensive pretreatment seems inappropriate for utilisation on single farms (Kusch et *al.*, 2011). Thus, there are obstacles for commercial scale of non-biological pretreatments of lignocellulosic biomass (Zheng et *al.*, 2009), except for grinding that is common in plants. It would exists around forty pilot-scale plants worldwide using diverse non-biological pretreatment such as steam explosion (personnal communication from R. Bakker, Wageningen University and Research). Full-scale non-biological pretreatment plants on crop residues are almost inexistant to our knowledges but a process called Economizer SE is commercially available (BiogasSystems, 2015). Non-biological pretreatments most likely to be applied at industrial scale (efficiciency, costs) are hydrothermal (steam explosion, hot water), chemical alkali ones (lime, ammonia, etc.) and grinding (Hendriks and Zeeman, 2009; Mönch-Tegeder et *al.*, 2014b). Among them, alkaline pretreatments are often highlighted for their efficiency and faisability (Carrere et *al.*, 2014; Chang et *al.*, 1998).

Concerning grinding, it must be noticed that usual grinding at industial scale are generally coarse. At laboratory scale, studied pretreatments correspond to a particule size of few millimeters (Monlau et *al.*, 2013). This kind of grinding is highly energy-consuming. For this reason, it is sometimes considered as not applicable at industrial scale (Hendriks and Zeeman, 2009). However, the applicability of mechanical disintegration pretreatment (median particle size ≈ 3 mm) was shown in a full-scale biogas plant with horse manure (Mönch-Tegeder et *al.*, 2014a, 2014b). The additional energy demand for mechanical pretreatment was considered as negligible

compared to the 26.5% increase methane production (Mönch-Tegeder et *al.*, 2014b). Thus, applicability of a pretreatment is dependent of process conditions and of the substrate.

Concerning chemical pretreatments, alkali ones may be less expensive and result in less cellulose degradation and high lignin degradation. Sodium hydroxide, lime (calcium hydroxide) and ammonia are common efficient alkalis used as pretreatment agents. In term of cost, sodium hydroxide is relatively expensive ($0.68/kg), ammonia is moderately expensive ($0.25/kg) and lime is inexpensive ($0.06/kg). Moreover, lime is safe to handle whereas NaOH and ammonia require careful handling (Chang et *al.*, 1998). The possibility to valorize digestate must also be considered. Again, CaOH (lime) appears as more interesting than NaOH. Calcium is necessary to plant and limits the counterproductive soil acidification whereas sodium increases counterproductive soil salinity. Another point to take into account with chemical pretreatments is the recycling and the possibility to recover chemicals. However, in anaerobic digestion it is not always necessary to wash pretreated substrate. Alkali can notably be helpful to limit acidification in AD reactors (C Sambusiti et *al.*, 2013) but sodium and calcium can both have an inhibitory effect on anaerobic digestion (from ≈ 4g/L) (Chen et *al.*, 2008). Finally, chemical pretreatments require high water amount to dilute reactives even if some investigations are carrying out to obtain solid-state pretreatment (Abdellatif Barakat et *al.*, 2014a).

Concerning thermo-chemical techniques, steam-explosion can be interesting for subsequent methane production (Monlau et *al.*, 2013) only if good parameters (temperature, duration…) are applied: too severe conditions can lead to condensation and precipitation of soluble lignin compounds and/or to an increase of cellulose crystallinity. Liquid hot water treatment is simple and not expensive but its action on lignin is minor (Hendriks and Zeeman, 2009). These pretreatments, with reduced exploitation costs, are interesting for an industrial application but the use of high pressure require important security rules that are sometimes difficult to apply in small plants.

In this context, biological pretreatments are promising (Zheng et *al.*, 2014). They include ensiling, partial composting, fungal and enzymatic pretreatments.

1.2.2 Biological treatments

Energy crops are usually stored by ensiling before anaerobic digestion. However, few studies have been carried out on the impact of such pretreatment. Ensiling consists of lactic fermentation (anaerobic) which converts sugars into acids (mainly lactic and acetic acids) and ethanol. Lignin is thus not degraded: generally, less than 6% without NaOH addition (Cui et *al.*, 2012; Herrmann et *al.*, 2011). High losses of cellulose and hemicelluloses can be observed: up to 21% after 83 days of wheat straw ensiling (Thompson et *al.*, 2005). Herrmann et *al.* (2011) measured organic matter loss ranging from 1 to 13% depending on: ensiling duration

(10 to 365 d), the feedstock (maize, sorghum, forage rye, triticale) and chemical (sodium nitrite and hexamethylene tetramine) or biological (*Lactobacillus plantarum* and *Lactobacillus buchneri*) additives. These organic matter losses led to an increase of the methane yield of ensiled biomass (up to 17%) but resulted to a little or no variation of the methane production reported to the initial biomass. With a mixture of grasses and ryegrass, ensiled as such or after drying, optimal ensiling conditions allowed preserving 96% of methane yield compared to substrate without storage. Under suboptimal conditions, up to 37% and 52% of CH_4 production were lost (Pakarinen et *al.*, 2008). Pakarinen et *al.* (2011) studied methane potential (L/kg VS) of several ensiled biomasses with no systematic measurements of dry matter loss during ensiling. They reported a 25 to 50% increase for hemp, 16% increase or 4% decrease for maize depending on duration and additives, and a decrease down to 34% for faba beans compared to fresh material. Even if ensiling is commonly used in farming, there are several parameters to optimize in order to maximize the subsequent methane production: initial moisture content, particles size, additives (chemical or enzymatic), etc. (Cui et *al.*, 2012; Ren et *al.*, 2006).

Partial composting has been scarcely investigated as pretreatment of anaerobic digestion. After 24 h pretreatment of the organic fraction of municipal solid wastes (OFMSW) with 2.5% v/v of mature compost, Fdez.-Güelfo et *al.* (2011) observed a 73% ($L/L_{reactor}$) increase of daily methane production in semi-continuous dry thermophilic anaerobic digesters. Brummeler & Koster (1990) carried out a pretreatment with a partial composting step (between 20 and 240 hours) on OFMSW. With SSAD batch (35% Total Solids, TS), they obtained up to 60% of the maximum methane production (determined in batch assays at low solid content) but with a 20% Volatile Solid (VS) loss. Without pretreatment those OFMSW did not produce methane in the same anaerobic digestion process which underwent acidification. Mshandete et *al.* (2005) obtained 26% higher methane yield with sisal pulp pretreated during 9 h under aerobic conditions. However, the obtained methane yield was lower than usual for this kind of biomass (Mshandete et *al.*, 2004). During aeration, there is a consumption of easily degradable compounds representing a loss of matter and so possibly less methane but this consumption can sometimes help to avoid acidification during the anaerobic digestion (Brummeler and Koster, 1990; Kusch et *al.*, 2008). Acidification can lead to an irreversible cessation of methane production.

Zhou and *al.* (2012) realized a stack-pretreatment with corn stover (5 cm). The pile was 1 meter high (5 kg/m^3) and let at ambient temperature for twenty days. The layer between 30 and 60 cm reached 52°C. It was then mixed with cow dung (1:1) and gave 30% more biogas than untreated material.

Whereas the above pretreatments are based on natural or uncontrolled complex microbial consortia, some authors have studied pretreatments with specific complex consortia. Zhong et *al.* (2011) used, on corn straw, a consortium containing yeasts (*Saccharomyces cerevisiae sp*., *Coccidioides immitis sp.*, and *Hansenula anomala sp.*), cellulolytic bacteria (*Bacillus licheniformis sp.*, *Pseudomonas sp.*, *Bacillus subtilis sp.*), a ligninolytic white-rot fungi (*Pleurotus florida sp.*), and the lactic acid bacteria *Lactobacillus deiliehii sp.*. A 15-day

pretreatment with 0.01% of this mixture allowed a 75% increase of methane production. Another route to obtain efficient microbial consortia consists in successive subcultivations (on PCS medium with lignocellulose addition) in order to enrich the consortium in lignocellulolytic microorganisms (Yan et *al.*, 2012). The resulting consortium was composed of *Bacteroidetes*, *Proteobacteria*, *Deferribacteres*, *Firmicutes*, *Lentisphaerae*, *Fibrobacteraceae* and other unidentified microorganisms. A 10-day pretreatment of rice straw with this consortium led to a 10% increase in batch methane production. Muthangya et *al.* (2009) realized a two-step fungal pretreatment with a four-day incubation with fungus CCTH-1 followed by eight-day incubation with the soft-rot *Trichoderma reesei* on sisal leaf decortication residues. Up to 101% of methane yield improvement was obtained per gram of pretreated substrate.

The most studied biological pretreatments of lignocellulosic biomass concern filamentous fungi pretreatment and enzymatic pretreatment or hydrolysis. In the case of enzymatic hydrolysis, most of the commercial enzyme cocktails are produced by fungi, in majority by ascomycetes *Trichoderma* and *Aspergillus* genus (Singhvi et *al.*, 2014). These cocktails are mainly composed of cellulases and xylanases. Their use requires a prior step to delignify the biomass and to make the holocelluloses accessible to enzymes. In comparison to fungi pretreatments, enzymatic pretreatments are in general, more complex and expensive as they require steps for enzyme production and extraction (Figure 1-14).

The first process scheme derives from the second generation bioethanol production which includes a physico-chemical pretreatment (Figure 1-14A). Although this step is mandatory in bioethanol process, it is optional in the case of use of ligninolitic enzyme extracts for anaerobic digestion. Enzymatic hydrolyse needs to be carried out under sterile conditions to avoid the consumption of released sugars by endogeneous microorganisms (Quéméneur et *al.*, 2012) before their conversion by anaerobic digestion. Another option is the introduction of enzymes in the digesters. It is not really a pretreatment because it occurs during the anaerobic digestion process but the goal is still to increase methane production. Although some enzymes are added in few full-scale anaerobic digester fed with maize silage, this process is not well referenced in litterature (Figure 1-14B). Several experiments were realized on this topic, principaly during anaerobic digestion of sillage but methane yield improvement rarely exceeded 20% (Brulé, 2014). This process also requires the costly production of enzymes extracts. Other biological techniques are used during the anaerobic digestion process like microaeration and two-stage digestion. For microaeration, small amount of oxygen is introduced in the reactor in order to allow aerobic or partial aerobic reactions like biogas desulphurization or hydrolysis improvement (Lim and Wang, 2013). To our knowledge, no methane yield improvements were measured for anaerobic digestion of lignocellulose with microaeration. Two-stage digestion consists in two reactors as already presented (see 1.1.5.2). Hydrolysis and methane production are improved with this reactor design. Moreover, hydrogen can be extracted and used as energy. However, the reactors cost is greater and must be considered. Finally, the simplest process scheme consists in the direct fungal pretreatment of lignocellulosic biomass before

its anaerobic digestion (Figure 1-14C). The number of steps of this process is minimum and it does not require any additional feedstock to produce fungi. Since fungi pretreatments are not expensive, they currently receive a growing interest.

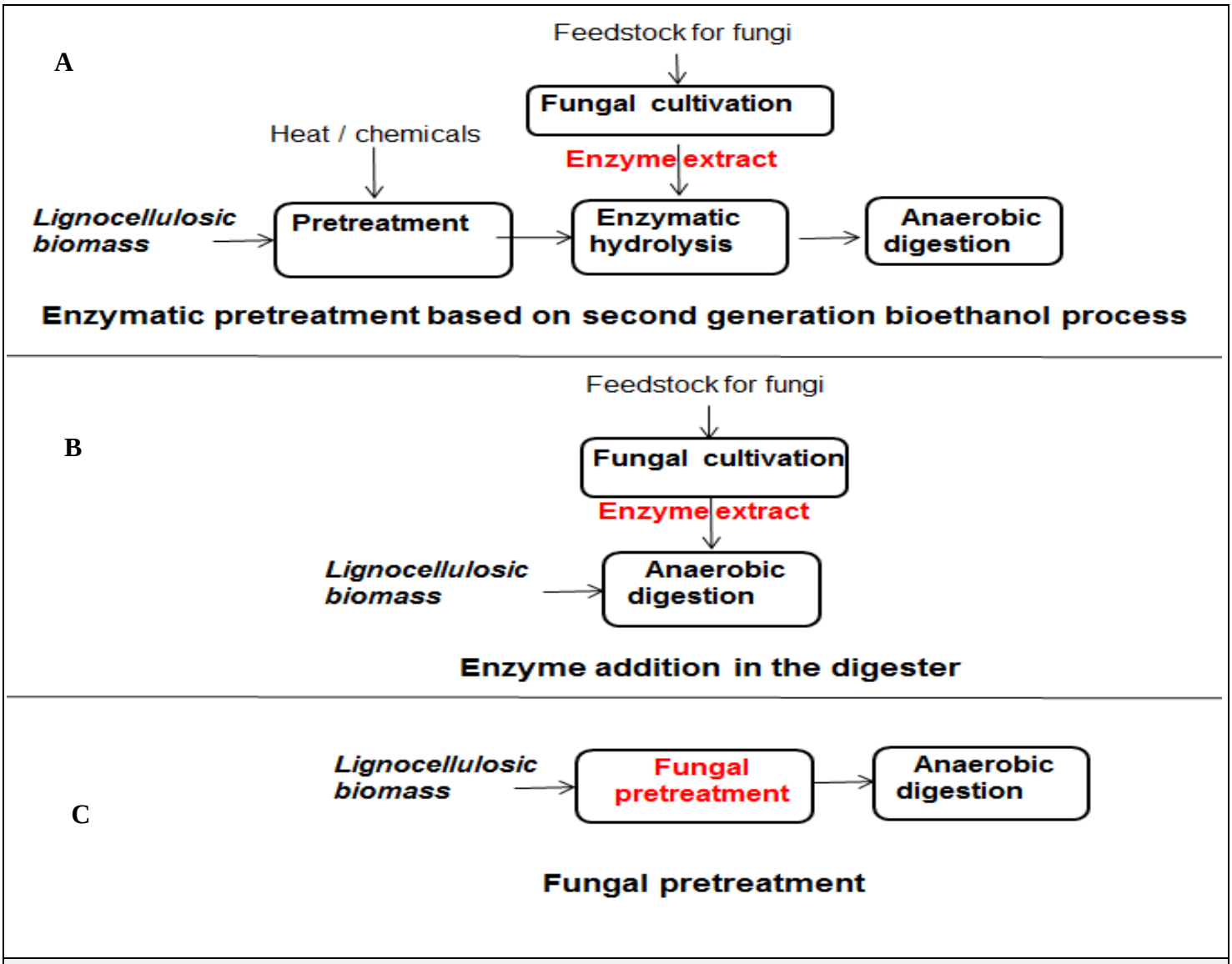


Figure 1-14. Strategies for the combination of enzymatic or fungal pretreatments and anaerobic digestion of lignocellulosic biomass.

Fungi that are able to degrade lignocellulosic biomass have been classified into white-, brown-, and soft-rot fungi. Degradation mechanisms by soft-rot fungi (asomycetes and deuteromycetes) are not well-known (Hammel, 1997). Sánchez (2009) reported that soft-rot fungi decrease Klason lignin in angiosperm wood and erode the secondary cell wall. However, they prefer substrate with low lignin content and degrade non phenolic structures. Brown rot fungi (BRF), basidiomycetes known to modify lignin, are able to attack cellulose and hemicelluloses with only small modifications on lignin (Eriksson et *al.*, 1990; Sánchez, 2009). Therefore, lignin is still a major component after fungal attack. The remaining lignin contains a greater number of ring

hydroxyl groups and is demethylated on arylmethoxy groups(Wan and Li, 2012). Conversely, white-rot fungi (WRF) are the most efficient organisms for delignification (Hatakka, 1983; Müller and Trösch, 1986; Wan and Li, 2012). They have a unique enzymatic system giving them the capacity to attack phenolic structures and to degrade lignin to CO_2 (Sánchez, 2009).

WRF give a bleached appearance to their substrate due to lignin removal. They belong to the *basidiomycota* phylum and, for a small part of them, to *Ascomycota* phylum (Hammel, 1997; Pointing et *al.*, 2003). Among WRF, the most rapid lignin degraders are basidiomycetes (Hammel, 1997; Sánchez, 2009). Some 'selective' strains of WRF lead to small loss of cellulose since they mostly use hemicelluloses as carbon source (Wan and Li, 2012). Sometimes, lignin tends to be removed faster than cellulose and hemicelluloses (Müller and Trösch, 1986; Salvachúa et *al.*, 2011) . WRF have a large field of action (Isroi et *al.*, 2011). They are able to decompose several aromatic pollutants or xenobiotics and thus can treat soil and water (Pinedo-Rivilla et *al.*, 2009). They are also used in paper industry to bleach kraft pulp (Ha et *al.*, 2001). Because of their ability to delignify, they were first investigated in order to improve forage digestibility. Lignin content and/or lignin linkages with holocelluloses are key parameters limiting biomass conversion into biofuels. Considering the obtained enhancement in forage digestibility or sugars yield, WRF are relevant to increase methane or bioethanol production.

WRF and ensiling both allow wet storage which reduces dry matter loss and fire risk and improves feedstock digestibility as compared with dry storage (Cui et *al.*, 2012). Cui et *al.* (2012) compared corn stover ensiling with or without NaOH addition and pretreatment with the WRF *Ceriporiopsis subvermispora*. Enzymatic hydrolysis yields were better with the WRF: until four times more released sugars (depending on operating conditions). Moreover, a 35-days pretreatment with *C. subvermispora* seemed sufficient whereas ensiling was carried out during 90 days. However, the WRF required an autoclave step for substrate sterilization.

The pretreatment with mature compost before anaerobic digestion gave good results (Fdez-Güelfo et *al.*, 2011). It is possible that WRF contributed to the increase of methane since they have been isolated during cooling and maturation phases (Pérez et *al.*, 2002). During main composting steps, WRF may not be involved in lignin degradation. To finish, even if other microorganisms than WRF can be interesting as pretreatment step for anaerobic digestion, they do not seem to outcompete WRF. Consortia (with WRF or not) can probably give better efficiency but they are also more difficult to control.

Notwithstanding, the use of WRF has also limitations, especially a long period (weeks to months) to provide an efficient pretreatment. Fungal pretreatment during storage can solve this issue (Cui et *al.*, 2012). Additional studies are also required to improve the selective degradation of lignin with WRF. Negative or positive results of the pretreatment step can be obtained depending on strains and cultivation parameters; and the establishment of inoculum is one of the main difficulties for WRF pretreatments (Cui et *al.*, 2012).

1.3 Enzymatic mechanisms of WRF and culture parameters

1.3.1 Enzymatic degradation by WRF and Solid-State Fermentation (SSF)

To attack lignocellulose, WRF use extra-cellular enzymes that form an hydrolytic system (hydrolases) and a ligninolytic system (Sánchez, 2009). The latter is composed by three major oxidizing enzymes: lignin peroxidase (LiP or 'ligninase' EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and laccase (or phenoloxidases, EC 1.10.3.2). Not all WRF have all these enzymes. They can have one or three of them or different paired combinations (Dashtban et *al.*, 2010; Tuor et *al.*, 1995). Other peroxidases were also reported: Versatile Peroxidase (VP, EC 1.11.1.16) (Martínez et *al.*, 2005), manganese independent peroxidase (MiP) (Moreira et *al.*, 1997), dye-decolorizing peroxidase (DyP) and aromatic peroxygenase (APO) (Liers et *al.*, 2011). Finally, several accessory oxidases are implied; some of them lead to H_2O_2 generation that is used by peroxidases. Accessory oxidases acts on several substrates: glyoxal, aromatic alcohols, etc. (Tian et *al.*, 2012).

Dashtban et *al.*(2010) schematized enzymatic processes of delignification (Figure 1-15). To sum up, laccase, LiP, MnP and VP are too bulky to penetrate cell walls. Thus, they oxidize small intermediates that will catalyze the pulling out of an electron from lignin aromatic rings (Tian et *al.*, 2012). Donors are organic acids, mediators and enzymes. The mycelia can also produce a non-enzymatic lignin degradation based on Fenton reaction. This leads to the production of free radicals that cleave cell-wall and favor the penetration of ligninolytic enzymes. Martínez et *al.* (2005) explained step by step chemical modifications on lignin with fungal enzymes. They reported diverse effects on lignin such as C4-ether breakdown, aromatic ring cleavage, Cα-Cβ breakdown or demethoxylation. They also pointed out that some lignin monomers can polymerize again or enter into the fungal hyphae to incorporate intracellular catabolic routes.

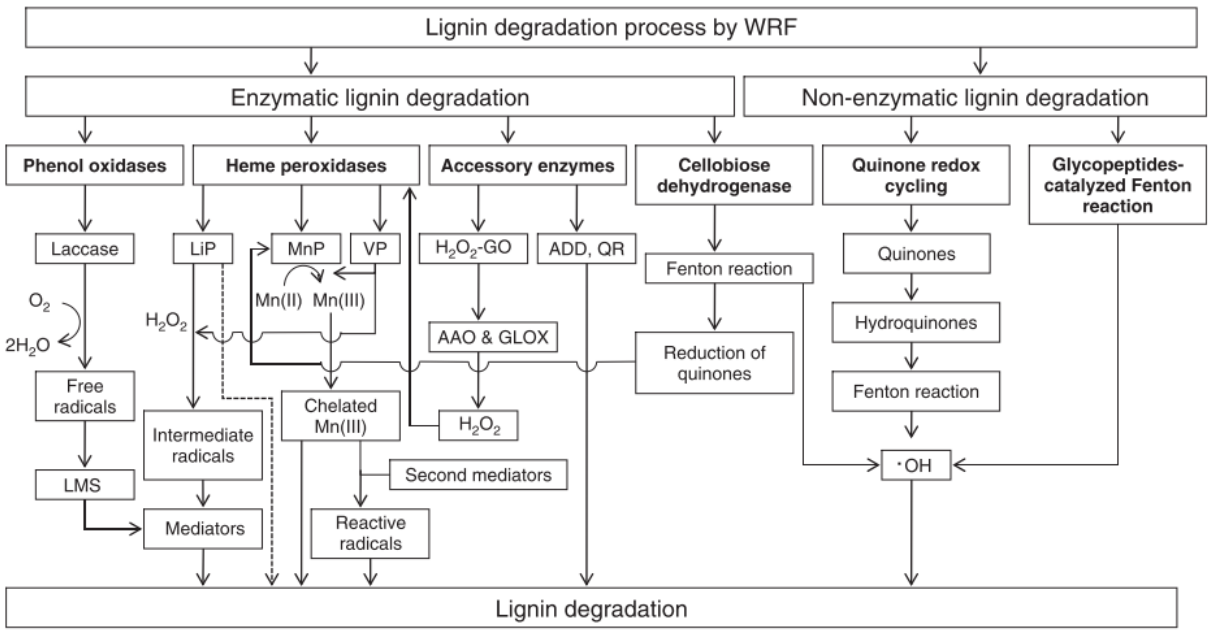


Figure 1-15. Schematic diagram of lignin degradation by white-rot fungi.
LMS: Laccase-mediator system. H_2O_2-GO: H_2O_2-generating oxidases. AAO: aryl-alcohol oxidase. GLOX: glyoxal oxidase. ADD: aryl-alcohol dehydrogenases. QR: quinone reductases. (Tian et *al.*, 2012)

Laccases and MnP act principally on phenols whereas LiP cleaves principally non-phenolic (lignin) compounds (up to 90% of the polymer). Under certain conditions, MnP can also act on non-phenolic moieties and so as laccases (Hatakka, 1994; Martínez et *al.*, 2005; Wan and Li, 2012). Even if it is not its principal function, it was reported that LiP can act on phenols in presence of veratryl alcohol (Camarero et *al.*, 1994). The three enzymes perform one-electron oxidation (Martínez et *al.*, 2005; Wan and Li, 2012). In contrast, VP is able to degrade phenolic and non-phenolic compounds (Martínez et *al.*, 2005). To bleach kraft pulp, MnP has a central role (Moreira et *al.*, 1997) and bleaching is related to lignin amount (Reid and Paice, 1994). Then, the important role of aryl alcohols (veratryl, etc.) must be noticed: stabilization of LiP, substrate for oxidases generating H_2O_2, etc. (Jong et *al.*, 1992).

Enzymatic equipment for delignification varies with fungi. Thus, the effectiveness of the pretreatment is dependent of the fungal strain and of the substrate but also of cultivation conditions. Solid cultures, better known under the name of solid-state fermentation (SSF), is an optimum way to cultivate fungi (Agosin and Odier, 1985; Tian et *al.*, 2012; Wan and Li, 2010). Several studies have shown that ligninolytic activity is more important with SSF culture than with liquid culture (Hölker et *al.*, 2004), perhaps because mechanisms are closer to those met in nature.

SSF is defined as a process in which substrate are decomposed by known microorganisms under controlled environmental conditions with the aim of producing a high quality standardized product. Substrate water content is low, generally between 1/1 and 1/10 water/substrate (Zadražil, 1993)

Advantages of SSF have been discussed by Tian et *al.* (2012). To summarize, much more substrate per volume unit is pretreated in solid process than in liquid culture. With SSF, the mycelia presence favors the attachment of enzymes to the substrate. Oxygen diffusion, necessary to fungal growth and lignin depolymerization, is greater. Moreover, SSF reactors design is less complex than those for liquid cultures and the process is cheaper (less aeration, agitation and heating). SSF also offers a less favorable environment for many bacteria and sterilization required energy costs are lower (Laskin et *al.*, 1992).

SSF with WRF, besides its practicality and its lower cost, is advantageous compared to enzymatic solutions. Modifying lignin, WRF increase its hydrophilicity (and thus its accessibility for hydrolysis). The mycelial penetration creating pores allows a greater available surface for enzymatic attacks. Inhibitors concentration is reduced owing to the fungal metabolism (Tian et *al.*, 2012).

1.3.2 Cultivation parameters for Solid-State Fermentation (SSF)

In their review, Wan & Li (2012) studied how to optimize the SSF parameters: inoculation, moisture content, temperature, aeration, particle size, supplements, and decontamination. For most of these factors, the optimum is substrate and fungal strain dependent.

Most WRF basidiomycetes are non-sporulating. Preculture in liquid medium, precolonized substrate or Cellophane Film Culture (CFC) can be used for inoculation. CFC was especially developed for SSF: low risk of inoculum contamination and less handling. Adequate amount of inoculum must be found to ensure good fungal growth and substrate colonization (Yoon et *al.*, 2014). Then, particle size (few centimeters) must be investigated to allow good exchanges (oxygen, water, metabolites) and hyphae penetration (Wan and Li, 2012).

For delignification, optimum moisture content is around 70 to 80% (Wan and Li, 2012) Optimum moisture content is function of the fiber retention capacity of the substrate (Reid, 1989). Thereby, for yard trimmings (Table 1-4), higher lignin degradation and lower cellulose loss were obtained at 60% than 75% (Zhao et *al.*, 2014b). A too high moisture limits oxygen circulation and substrate loading (Wan and Li, 2012) but high moisture content can favor ligninolytic enzymes production (Isroi et *al.*, 2011). It is likely caused by the transferring nutrients role of water (Zhao et *al.*, 2014a). Sufficient water is thus required for fungal growth (Reid, 1989; Wan and Li, 2012). Moisture content can sometimes decrease during SSF (Reid, 1989; Zadražil and Brunnert, 1982). In contrast, a significant increase in moisture content can be observed with fast-growing fungi because fungal metabolism generates water.

Decontamination of the reactor and inputs (water, air and substrate) is an important parameter especially for process scale up. However, Akin et *al.* (1995) observed no difference in the in vitro digestibility by rumen microorganisms with or without endogenous microflora of Bermuda grass stems. Some fungal strains are more tolerant to some contaminations. Endogenous flora can sometimes inhibit fungal growth (Pointing, 2001; Reid, 1989). It is possible to partially decontaminate with cheap techniques (steam, salts, solar heat pasteurization…) (Wan and Li, 2012). Zadražil & Puniya (1995) suggest that moderate acidic pH would favor delignification and limit bacterial contamination, WRF have a natural tendency to decrease substrate pH. On wheat straw Zadražil &Peerally (1986) obtained similar results with a short anaerobic fermentation step (50°C for 48h) before inoculation with the desired fungus as with a sterilization step (Zadražil, 1993). Comparing several disinfection methods, Ficior et *al.* (2006) showed that a dilute aromatic fungicide (Derosal 0.01%) can also be a successful way to disinfect the substrate. Even if fungal metabolism could be affected by disinfection, it must be noticed that fungal yield can be very low without decontamination (Ficior et *al.*, 2006).

Pre-treatment duration can last for weeks or months, it is a function of fungal growth rate and of the substrate recalcitrance (Wan and Li, 2012). It seems that during the substrate colonization, the digestibility decreases before increasing during fungus maturation (Zadražil, 1993). The decrease can be due to water soluble

substances consumption (Zadražil, 1977). Cereal straw can be totally mineralized in approximately 90 days (Zadražil, 1985) although the treatment generally lasts between two and four weeks (Table 1-5).

For basidiomycetes, optimum temperature for delignification is around 25°C to 30°C. Fungal metabolism produces heat that sometimes leads to temperature gradients in the solid substrate. A too high temperature can kill the selected fungi and heat dissipation must be provided for full-scale plants (Wan and Li, 2012). According to Zadražil (1993), a too high temperature (regarding optimum temperature for fungi) favors contamination. The optimum temperature is fungi dependent. Higher temperature did not always lead to increased organic matter (OM) decomposition rate even with a long pretreatment period (60 days). In addition, enhanced OM decomposition did not always lead to a better anaerobic degradability (Zadražil, 1985) since OM decomposition must be selective (lignin, high crystalline structures…).

Oxygen is essential for WRF growth and for the oxidative process of delignification. Oxygenation often enhances ligninolytic enzymes production (Isroi et *al.*, 2011). Thus, delignification is sometimes better with greater oxygenation (Kamra and Zadražil, 1986 with Pleurotus eryngii; Kirk et *al.*, 1978 with Phlebia Tremellosa) but this parameter is strain dependent (López et *al.*, 2002). As reviewed by Reid (1989), selective delignification was not increased with higher oxygenation. However, a better reducing sugars yield after hydrolysis can be obtained under oxygen atmosphere (Hatakka, 1983). Delignification is enhanced by oxygen through the production of hydrogen peroxide. However, hydrogen peroxide comes from the co-metabolism of cellulose and/or hemicelluloses (Sánchez, 2009). This phenomenon could explain the lower selectivity for lignin degradation. Since the pre-treatment duration can sometimes be reduced with oxygenation (Hatakka, 1983), it must be taken into account as well as the additive cost. Furthermore, aeration is function of the substrate packing behavior and of its porosity. Finally, there is an interdependence with other parameters (heat dissipation, water distribution…) (Isroi et *al.*, 2011).

Concerning supplements, several have been tested to improve fungal pretreatments (N, Mn…). Firstly, the effect of supplements can be substrate dependent as with wood chips where diffusion is difficult and where supplements increase fungal biomass and colonization in depth (Messner et *al.*, 1998). Secondly, results vary between liquid and solid cultures (Wan and Li, 2012), they are also function of other parameters such as agitation for liquid cultures (Khiyami et *al.*, 2006) or culture duration. Finally, beneficial or negative effects are function of the concentration used (Shrestha et *al.*, 2008b). Several indicators were measured to evaluate the effect of supplementation during fungal pretreatment such as Klason lignin reduction, glucose yield or ligninolytic enzymes production. However, for anaerobic digestion applications these indicators are not sufficient to assess the efficiency of the pretreatment. Therefore, caution is required to compare the performed studies.

It must be noticed that for most WRF, delignification is induced under nitrogen starvation. Jung et *al.* (1992) hypothesized that lignocellulosic substrate with high protein content (like alfalfa) can limit delignification with fungal treatment. Nitrogen supplementation can inhibit delignification while stimulating fungal growth and sugars consumption (Wan and Li, 2012). Some studies reported a positive effect of N supplementation on ligninolytic enzymes production with sometimes an influence of pH and N source (Isroi et *al.*, 2011). This higher enzymatic production cannot be linked to a better delignification. During biobleaching, Moreira et *al.* (1997) hypothesized that the limiting factor can be the production of intermediates, especially H_2O_2, instead of enzymatic production. The lack in selectivity under high nitrogen medium can be linked to a stimulation of cellulose consumption under those conditions. According to Mikiashvili et *al.* (2006) better ligninolytic enzymes accumulation in the medium was caused by higher fungal biomass.

Mn^{2+} addition was shown to be beneficial in several studies (Khiyami et *al.*, 2006; Salvachúa et *al.*, 2013; Shrestha et *al.*, 2008b), probably because of its cofactor role for MnP and VP (conversion of Mn2+ to Mn3+) (Wan and Li, 2012). Mn^{3+} acts as a starter of lipid peroxidation reactions and as an oxidizer of phenolic structures (Monlau et *al.*, 2013). In addition, Mn2+ has a regulatory role on MnP gene (Isroi et *al.*, 2011). Copper and iron can improve delignification by WRF (Salvachúa et *al.*, 2013), they are present in laccase and LiP, respectively. Copper increases transcription and activity of laccase (Shah et *al.*, 2010). Hydrolytic enzymes production was significantly decreased with nanoparticles (of iron and copper) under submerged fermentation conditions whereas it was enhanced with ionic forms (Shah et *al.*, 2010). Ethanol was successfully used as laccase inducer (Meza et *al.*, 2006). Some improvements were also reported with concomitant H2O2 and veratryl alcohol supplementation (Khiyami et *al.*, 2006). Phenolic compounds can enhance substrate degradation and fungal growth if combined with sugars (Platt et *al.*, 1983).To our knowledge, no supplementation was done to a fungal pretreated substrate followed by anaerobic digestion. After the pretreatment, if the remaining concentration of added nutriment is sufficient, it may act as useful oligoelements for anaerobic microorganisms (Angelidaki et *al.*, 2003) or may have an inhibitory effect, especially with heavy metals (Lin, 1992).

To conclude, SSF is a complex process to monitor and to optimize. Several culture parameters are interdependent. Best conditions for fungal growth are not always best conditions for delignification.

1.4 Quantification of anaerobic digestibility improvement after WRF pretreatment

1.4.1 Anaerobic digestion studies with WRF pretreatment

Several numeric data are given by Isroi et *al.* (2011) regarding hydrolysis yield or lignin loss. However, few studies use WRF to improve methane yield or rate. Among them, some are based on the depolluting action of WRF to reduce inhibitors of anaerobic digestion like limonene or phenols (Dhouib et *al.*, 2006; Fountoulakis et *al.*, 2002). Thus, a small number of publications related the beneficial delignification effect of a pretreated substrate for methane production (Table 1-4). On average, it seems possible to improve methane yield by 50% with a pretreatment period of 30 days. Several authors observed not only an increase of the biogas production but also a higher content of methane. When the pretreatment increases the anaerobic degradability, the prior production of Volatile Fatty Acids (VFA) is sometimes augmented (Jalč et *al.*, 1997; Karunanandaa and Varga, 1996a; Müller and Trösch, 1986). Fungal pretreatment seems particularly effective on woody biomass with high lignin content (Table 1-4). This biomass has a little methane potential and the effect of the pretreatment is more visible. It can also be noticed that some fungi are well-known and more studied like *Pleurotus florida* or *Phanerochaete chrysosporium*.

The enhancement in methane production must be compared to the loss of substrate during the pretreatment period. Improvement of methane production is often expressed in terms of volume per weight of pretreated substrate although this weight is inferior to the weight of untreated sample. Dry matter losses lead to losses of methane potential therefore these losses must be compensated by sufficiently higher methane potential for pretreated substrate. Notwithstanding, measurements of dry matter losses due to the pretreatment are rare for anaerobic digestion studies. If carbohydrates losses are too high, the pretreatment can impact negatively methane production (López et *al.*, 2013). Another possible benefit of fungal action is the enhancement of anaerobic digestion kinetics (Müller and Trösch, 1986).

1.4.1.1 Nitrogen metabolism for anaerobic digestion of fungal pretreated substrates

Dry crop residues are rich in carbon. Their C/N ratio is often superior to the optimum value for anaerobic digestion that is between 20 and 35 (Angelidaki et *al.*, 2003; Sialve et *al.*, 2009). To obtain a better C/N ratio and to enhance biogas production, Zhou et *al.* (2012) codigested biological pretreated corn stover with cow dung. Moreover, the source of N supplementation for anaerobic digestion has an influence on the process. Cow dung is already rich in ammonia-N whereas proteins should be hydrolyzed to be converted in ammonia-N. A combination of concentrate feed (11% crude proteins) and fungal treated rice straw led to a difficult nitrogen utilization by ruminal microorganisms (Karunanandaa & Varga, 1996b). Digestion of crude proteins was lower compared to same diet but with untreated straw. However, the digestibility for the pretreated straw was improved even if the low concentration of ammonia-N can have possibly limited the anaerobic digestion.

Cellulolytic bacteria require isoacids and ammonia-N but none of these components was present in the pretreated diet. It is thus possible that there was stimulating molecules for cellulolytics generated during Solid-State Fermentation (SSF) by WRF: for example 3-phenylpropanoic acid derived from lignin degradation (Eggeling, 1983).

Fungal pretreatment can increase the content of N (Bisaria et *al.*, 1983; Zeng et *al.*, 2011) and crude proteins (Jalč et *al.*, 1997) reported to dry matter. However, there is dry matter loss during the pretreatment. Without loss of nitrogen, if analyses are expressed in percentage of dry matter, an artifactitious increase could be observed because N amount is reported to the dry matter amount. The augmented protein content can also result from fungal biomass (Jalč et *al.*, 1997). Agosin et *al.* (1985) found that mycelial content rarely exceed 10% of wheat straw TS in 18 days or more. Fungal pretreatment can modify the amino acids composition of the substrate (Valmaseda et *al.*, 1991). Biodegradability and anaerobic digestibility of proteins vary according to their compositions (Jimenez et *al.*, 2014).

1.4.1.2 Slight acidification of pretreated substrates

In general, WRF lead to a pH drop (Jalč et *al.*, 1997; Zadražil, 1985). Agosin et *al.* (1985) observed approximately one pH unit less in five days. Moderate low pH values favor ligninolytic enzymes production. Agosin & Odier (1985) stated that the more ligninolytic the fungus, the lower the substrate pH. Lignin degradation products probably have a role in this pH drop even if Borneman et *al.* (1986) did not see a pH drop with addition of phenolic monomers until 10 mM. The release of acetyl groups during the solubilization of lignin and hemicelluloses can also be responsible for pH drop (Cui et *al.*, 2012). During pretreatment, there is also oxalic acid production associated with MnP activity and acting as chelator of produced Mn^{3+} (D'Annibale et *al.*, 1998; Tian et *al.*, 2012). Moderate low pH can sometimes contribute to limit contamination but pH should not be too low for anaerobic digestion (near neutrality). Thus, Müller & Trösch (1986) added cow manure in the anaerobic reactor to avoid acidification, they tested 50%, 33% and 25% addition of straw to cow manure and retained 33% straw (in dry matter) (Table 1-4).

Thus, co-digestion with cow dung was sometimes required with different goals: to obtain an optimal C/N ratio and to avoid acidification. For anaerobic digestion, cow dung can also play the role of inoculum.

Table 1-4 (part 1/2). Improvement of anaerobic digestion of pretreated lignocellulosic substrates by WRF.

Substrate		Fungi (* = selected after screening)	Additional information about fungal process used	Anaerobic digestion process	Enhancement in methane or biogas per weight of pretreated substrate	References
EFFLUENT	Olive Mill Wastewaters (OMW)	*Phanerochaete chrysosporium*	Removal of toxic phenolic compounds in OMW	**Continuous** at mesophilic temperature, mean loading rates varied from 2 to 8 g COD /L/d, hydraulic retention time (HRT) of 3–5 days	50% (v/ w COD) CH_4 more	(Dhouib et *al.*, 2006)
	Kraft pulp mill effluents	*Phanerochaete chrysosporium*	Pretreatment by mycelial pellets or extracellular ligninolytic liquid	**Batch** at mesophilic temperature (nutrient addition, adjusted pH)	Enhancement of the anaerobic degradation of high molecular weight compounds by 30%	(Feijoo et *al.*, 1995)
GRASS AND STRAW	Grass hay	*Pleurotus ostreatus*			4% CH_4 more	(Lehtomaki et *al.*, 2004)
	Rice straw	*Coriolus versicolor*	5 d at 27°C	**Batch** at mesophilic temperature for 35 d ***Co-digestion with cattle dung***	35% BIOGAS (v/w VS)	(Phutela et *al.*, 2011)
		Phanerochaete chrysosporium (a brown-rot also tested)	21 d with oligoelements ($FeSO_4$…) and an autoclaved substrate	**Batch** at mesophilic temperature during 63 d	46% (v/w TS) CH_4 more	(Ghosh and Bhattacharyya, 1999)
		Pleurotus sajor-caju	40 d of growth	**Batch** at mesophilic during 15 d	≈59% (v/w) CH_4 more	(Bisaria et *al.*, 1983)
	Wheat straw	*Pleurotus florida* * (22 basidiomycetes)	90 d, **SSF**, 75% water content for sterilized wheat straw at 25°C, inoculated with mycelium grown for 10 days on malt-extract agar slants.	**Batch** ***In combination with cow manure*** (2:1 straw TS) to avoid acidification (less addition of NaOH) 30 d 37°C	27.9% (v/w TS) *42.5% TS loss*	(Müller and Trösch, 1986)
	Cyamopsis tetragonoloba	*Pleurotus sajor-caju*	40 days (d) of growth	**Batch** at mesophilic temperature	≈63% (v/w) CH_4 more	(Bisaria et *al.*, 1983)
	Corn stalk	*Pleurotus florida*	30 d 75% moisture content with sterilized corn stalk	**Batch** reactors at mesophilic temperature with three initial solid loadings	≈38% BIOGAS (v/w)	(Dongyan et *al.*, 2003)
	Miscanthus	*C. subvermispora (ATCC 96608)*	28d, 50% moisture content at 28°C, autoclaved substrate	**Solid state anaerobic digestion** (20% TS content), **batch** 60 d 37°C	+25% (v/ w VS) CH_4 *≈20% TS loss*	(Vasco-correa and Li, 2015)

Table 1-4 (part 2/2). Improvement of anaerobic digestion of pretreated lignocellulosic substrates by WRF.

Substrate		Fungi (* = selected after screening)	Additional information about fungal process used	Anaerobic digestion process	Enhancement in methane or biogas per weight of pretreated substrate	References
WOODY BIOMASS	Japanese cedar chips	*Cyathus Stercoreus* AW 03-72 (Cster) *Trametes hirsuta* AW 03-72 * (Th) (6 fungi)	20 d at 37°C	**Batch** at mesophilic temperature	43 mL CH_4/g TS instead of 0 for Cster 30 mL CH_4/g TS instead of 0 for Th	(Take et *al.*, 2006)
	Japanese cedar wood	*Ceriporiopsis subvermispora* (Csub) and *Pleurocybella porrigens*	56 d	**Batch** at mesophilic temperature	600% (v/w) CH_4 more with *Csub* CBS 347.63 strain *TS loss 3.1%*	(Amirta et *al.*, 2006)
	Lignocellulosic biomass (grass, corn stover, wheat straw and wood fiber)	*Phanerochaete flavido-alba*	21 d at 30°C with nutrients (glucose…) and an autoclaved substrate	Thermophilic **batch**	76 NmL CH_4/g TS for wood fiber instead of 0	(López et *al.*, 2013)
	Castanea sativa	*Auricularia auricula-judae*	4-5 weeks at 37°C	**Continuous** at mesophilic temperature	15% CH_4 more	(Mackuľak et *al.*, 2012)
	Yard trimmings	*Ceriporiopsis subvermispora*	30 d at 28°C and 60% initial moisture content with autoclaved substrate	**Solid-state batch** at mesophilic temperature	106% (v/w VS) CH_4 more than the control group (autoclaved without inoculation) and 154% (v/w VS) higher than raw yard trimmings *TS loss 12,5%*	(Zhao et *al.*, 2014b)
	Albizia Chips	*C. subvermispora (ATCC 96608)*	48 d 28°C, 60% moisture content, autoclaved substrate	**Solid-state batch** (20% TS content) at mesophilic temperature 58 d	370% (v/w VS) CH_4 *≈10% TS loss*	(Ge et *al.*, 2015)

1.4.2 *In Vitro* Dry Matter Digestibility (IVDMD) studies

Not so much data are available about the use of a WRF pretreated substrate in anaerobic digestion reactors. In contrast several studies were carried out to evaluate *in vitro* digestibility. The idea to use white-rot fungi to improve the digestibility of lignocellulosic biomass for ruminants dates of 1902 by Falck (Cohen et *al.*, 2002).

The determination of the *In Vitro* Dry Matter Digestibility (IVDMD) is another way to access the anaerobic biodegradability of a pretreated substrate. This method (Tilley and Terry, 1963) typically consists in a 48 h digestion of the substrate with rumen fluid followed by an acid pepsin digestion. The original method was established with 146 samples of gramineae and legumes, it was improved by Marten & Barnes (1980). In order to increase digestibility for animal feeding, a lot of authors have used the IVDMD to study the impact of a fungal pretreatment on lignocellulosic biomass.

Tilley & Terry (1963) found a good correlation between IVDMD and *in vivo* digestibility in the rumen. It is not always the case, especially for substrates with high post-rumen digestion like acid substrates (maize silage…) (Adesogan et *al.*, 1998). Another way to evaluate the *in vitro* digestibility consists in measuring the gas production of a substrate digested with rumen fluid during few hours to few days (Blümmel and Ørskov, 1993; Khazaal et *al.*, 2010; Menke et *al.*, 1979). The link between an industrial anaerobic reactor and anaerobic digestion with rumen flora is not very obvious in term of methane production. First, substrates have more time to be degraded in biogas plant and thus potentially produce more biogas. Cellulose can only be partially degraded in the rumen compared to degradation in a biogas plant (25% to 50% less) (Amon et *al.*, 2007). Second, VFA are absorbed by the rumen epithelium *in vivo*. Those ones are no more available for the transformation into methane. Bacterial populations of rumen and biogas plant are different. In the rumen, there are more hydrogenotrophic methanogens (H_2/CO_2 substrate) and not many acetoclastic methanogens. However, anaerobic digestion of lignocellulosic materials in reactors seeded with rumen microorganisms was efficient (Hu and Yu, 2006). Raju et *al.* (2011) failed to find a good correlation between IVDMD and methane potential for meadow grasses ($R^2 = 0.41$). As part of digested matter is converted to CO_2 with anaerobic digestion, the study of the correlation between biogas production and IVDMD would be interesting. Chynoweth et *al.* (1993) compared BMP and IVDMD assays. They observed a similar disappearance of suspended solids in both process but biogas production was not directly linked to this disappearance. Thus, a part of the solubilized matter was lost (handling error) or converted into refractory compounds. Therefore IVDMD studies on fungal pretreated biomass may give information on the behavior of theses substrate during anaerobic digestion.

As for anaerobic digestion, several parameters influence IVDMD. Jalč et *al.* (1997) observed an improvement of the IVDMD pretreating the substrate with a strain unable to delignify it (*lnonotus dryophilus*). Therefore, biodegradability is not only dependent on the lignin content. At the beginning of fungal growth, a decrease in IVDMD is observed, then there is a rapid improvement (Agosin and Odier, 1985; Jalč, 2002; Zadražil, 1993). Studies on white-rot pretreated feed were grouped together by Jalč (2002) and Isroi et *al.* (2011) with positive and negative impacts on IVDMD. Depending on the pretreatment, the IVDMD can be decreased, especially if fermentable carbohydrates are consumed (Zadražil, 1985). In Table 1-5 are reported some positive results given by Jalč (2002) and Isroi et *al.* (2011) and by others. As for biogas plants, the total solids (TS) loss must be taken into account. Pretreatment periods vary from 20 to 120 days and the average TS loss is around 20% (Table 1-5). One of the highest TS loss was 52% in five weeks (Hatakka, 1994). Jalč (2002) in an extensive review on straw degradation by white-rot fungi, noted that average TS loss is normally about 26% for wheat straw with values varying from 12% to 66% (Rodrigues et *al.*, 2008). Average IVDMD improvement by WRF pretreatment varied between 10 and 40% (Table 1-5). Some IVDMD increases were higher than 80% with less than 20% TS loss (Agosin et *al.*, 1985; Akin et *al.*, 1993b). In Table 1-5, the increased digestibility of a substrate coming from a SSF pilot scale process (5 kg) should be noticed (Karunanandaa et *al.*, 1992). With a large scale experiment (1500 kg of treated wheat straw by SSF), IVDMD was also improved (Zadražil, 1993).The process used can be optimized: for example by diminishing the thickness of the wheat straw layer (between 1.5 and 2 m in the experiment) to favor transfers (water, O_2…).

Table 1-5 (part 1/2). Improvement of IVDMD by fungal pretreatment of lignocellulosic biomass.				
Substrate	Fungi (WRF) (* = selected after screening)	Additionnal information about fungal culture	Improvement percentage of IVDMD compared to control [a]	References
WHEAT STRAW	*Grifola frondosa* (Gf) *Dichomitus squalens* (Ds) *Inonotus rheades* (Ir) *Pleurotus liguatilis* (Pl)* (235 strains belonging to 150 species)	sterilized wheat straw (25 g < 1mm + 75 mL deionized water) pretreated at 22°C, 25°C and 30°C during 30 and 60 d	33.5% for Gf, 25°C, 60 d 32.2% for Ds, 22°C, 60 d 36.1% for Ir, 30°C, 60 d 31.6% for Pl, 30°C, 60 d ΔIVDMD at 60 d: -32 to +36% for all tested strains *TS loss between 0% to 118%*	(Zadražil, 1985)
	Cyathus stercoreus (Cs) *Pycnoporus cinnabarinus* (Pci)	SSF with ground autoclaved wheat straw ≈18 d at 30°C for Cs and 37°C for Pci under 100% oxygen	94% for Cs *TS loss -18%* 63% for Pci *TS loss -12%*	(Agosin et *al.*, 1985)
	Cyathus stercoreus (Cs)* (from 8 strains)	20 d	78% for Cs *-15-20% TS loss*	(Agosin and Odier, 1985)
	Stropharia rugosoannulata (Sr) (from 4 strains) *Pleurotus cornucopiae* (Pco)	30°C 120 d for Sr 22°C 120 d for Pco	31.6% for Sr ≈ *-60% OM loss* 19.8% for Pco≈ *-35% TS loss*	(Zadražil, 1977)
	Pleurotus sp.	1500 kg of wheat straw during 38 d	13.8% more in average	(Zadražil, 1993)
	*Peniophora utriculosa** (72 brazilian tropical fungi)	30 d at 25°C or 30°C 25 g milled sterile straw (<1mm) in 75 mL deionized sterile water	36% more	(Capelari and Zadražil, 1997)
	Hericium clathroides(Hc) *lnonotus dryophilus (Id)* (from 6 strains)	SSF, 30 d at 28° C	35% IVDMD *12% TS loss for Hc and 24% for Id*	(Jalč et *al.*, 1997)
	Phanerochaete chrysosporium (PC), *Scytinostrorna galactinum*, *Phlebia tremellosa*, *Phellinus pini*, and *Pholiota mutabilis*	30 d at 28° C and 90% relative humidity	26.4% only PC increased IVDMD of oat straw *42.3% TS loss (11.7% for control)*	(Jung et *al.*, 1992)

[a] = 100-(IVDMD of pretreated substrate * 100/ IVDMD of the control)

Table 1-5 (part 2/2). Improvement of IVDMD by fungal pretreatment of lignocellulosic biomass.

Substrate	Fungi (WRF) (* = selected after screening)	Additionnal information about fungal culture	Improvement percentage of IVDMD compared to control [a]	References
Oat straw and alfalfa stems	*Cyathus stercoreus* (Cs) *Phanerochaete chrysosporium* (PC) *Pleurotus sajor-caju*	SSF (**5 kg** batches), 30 days, initial moisture content 75-80%, sterilized straw (5-15cm)	42% for Cs	(Karunanandaa and Varga, 1996a)
Rice straw leaf and stem	*Cyathus stercoreus* (Cs) *Phanerochaete chrysosporium* (PC) *Pleurotus sajor-caju*	**50 g** TS autoclaved in 150 mL water in flasks stoppered with cotton plugs during 30 d	29% for Cs on leaf (lesser digestibility for stem richer in silicates) *≈10% Loss of Organic Matter*	(Karunanandaa et *al.*, 1995)
Rice straw, Corn stover	*Dichomitus squalens* and two strains (cellulase-less mutant and a wild type) of *Phanerochaete chrysosporium*	SSF 30 d (better improvement than 15 d) at 25°C sterilized straw	45.8% for Cs on rice straw TS loss = 6.8% 38.2% for Cs on corn stover TS loss = 3.3%	(Karunanandaa et *al.*, 1992)
Bermuda grass	*Cyathus stercoreus* (Cs) *3 Phanerochaete chrysosporium* *Phellinus pini*	sterilized stem sections (10-mm-long) in agar plates during 6 weeks at 39°C for PC and 27°C for others	80% for Cs *TS loss 18%*	(Akin et *al.*, 1993b)

[a] = 100-(IVDMD of pretreated substrate * 100/ IVDMD of the control)

1.5 Parameters of lignocellulosic biomass influencing its anaerobic degradability

1.5.1 Polymers composition and enzymatic accessibility

A predictive model for methane production allowed classifying some parameters of lignocellulosic biomass: there was a high negative influence of lignin content and a small negative influence of crystalline cellulose content, a positive influence of soluble sugars content (measured after mild acid hydrolysis) and, to a lesser extent, a positive influence of crude protein and amorphous holocelluloses contents (Monlau et *al.*, 2012b).

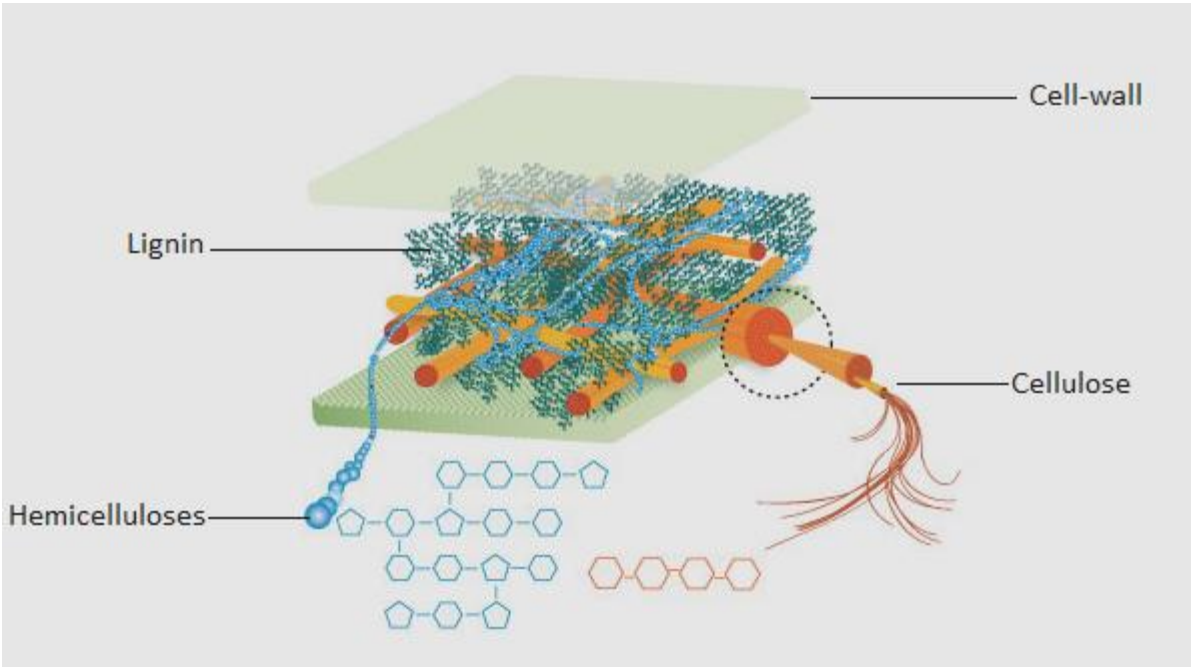
In addition, Monlau et *al.* (2013) inventoried the main parameters influencing anaerobic digestion of lignocellulose. This latter is limited by the hydrolysis step. Thus, the accessibility of the substrate to enzymatic attacks is primordial. Pore volume influences the digestibility (must be high). G/S (guaiacyl/synapyl) ratio influences lignin degradability. G units lead to condensed structure (C-C bounds) with little biodegradability whereas lignin S units are easier to degrade anaerobically (Abdellatif Barakat et *al.*, 2014b). Crystalline cellulose is more recalcitrant than the amorphous form during anaerobic digestion, so crystalline index must be low. The higher the ratio xylose/arabinose (X/A), the better the hemicelluloses digestibility (A Barakat et *al.*, 2014). The substitution of xylose by arabinose makes the structure more complex and decreases the digestibility. Hemicelluloses and cellulose are linked by H bonds (Sun, 2010). Some hemicelluloses contain acetyl groups contributing to those linkages (in graminaceous, for example). Acetyl groups removal led to a better hydrolysis of cellulose and xylan (Kong et *al.*, 1992; Zhang et *al.*, 2011). Those groups are partly responsible for the restricted degradation of forages in the rumen. Xylan degradation is difficult prior to the removal of lateral substituents. Finally, the hydrolysis of the complex lignin-carbohydrates is the main obstacle to anaerobic degradation.

1.5.2 The lignin-carbohydrates complex (LCC): linkages and derived compounds

1.5.2.1 Major lignin linkages in cell-wall: ferulic acid (FA) and p-coumaric acid (pCA)

Cross-linkages (both between lignin and carbohydrates and within lignin) enhance the rigidity, complicate the structure and limit the availability of carbohydrates to anaerobic microorganisms (Jalč, 2002) (Figure 1-16). Major obstacles to the biodegradability of lignocellulosic biomass remain the cleaving of LCC bonds and the removal of lignin (Monlau et *al.*, 2013). Complete removal of lignin is not necessary for important glucose released after enzymatic hydrolysis and cleavage of bonds between lignin and cellulose would be sufficient (Brudecki et *al.*, 2013). The level of delignification required may depend on the lignocellulosic matrix considered (species, etc.). It exist two biosynthetic pathways to build LCC (nucleophilic attack or oxidative coupling), but even if they modify the structure, they have no effect on degradability (Boukari et *al.*, 2009; Grabber, 2005; Monlau et *al.*, 2013).

A



B

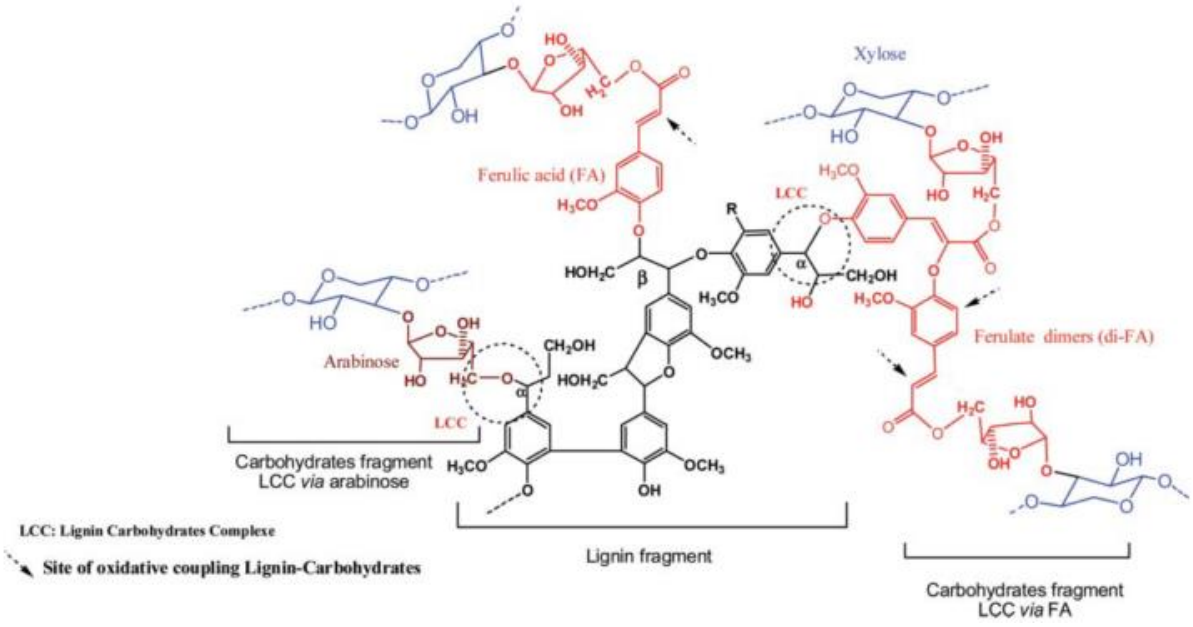


Figure 1-16. Lignin-carbohydrates Complex (LCC)

A. Schematic representation of cell-wall and its main polymers (Adapted from Ceres, 2007). B. Representation of linkages between lignin and carbohydrates via phenolic acids and arabinose in non-woody plants (Monlau et *al.*, 2013).

Lignin monomers are linked together by β-O-4 aryl ether linkages in majority (Annex 1). Uncondensed units linked in this way are preferentially degraded. However, Agosin et *al.* (1985) failed to find a direct correlation between the cleavage of these bounds and digestibility improvement (IVDMD). Thus, other linkages are important to cleave like lignin-carbohydrates bounds Monlau et *al.* (2013). Indeed, lignin is linked to hemicelluloses, limiting their hydrolysis. These linkages are in majority phenolic acids ester-linked (1-2% of cell walls, Annex 1) to arabinose. Ferulic acid (FA) is one of them and is also ether-linked to lignin (hemicelluloses-ester-FA-ether-lignin bridges). In gramineae, hemicelluloses can also be bound to lignin via

diferulic acids (Figure 1-16B). Recent studies have also concluded that FA is an intrinsic part of the lignin structure in grasses (Río et *al.*, 2012) . Then, p-coumaric acids (pCA) can sometimes have a lignin/hemicelluloses cross-linking function but lots of pCA are also ester-linked inside lignin molecules (Sun, 2010).

1.5.2.2 Anaerobic degradability of FA and pCA

The impact of FA and pCA on anaerobic digestion is not very clear (Yosef and Ben-Ghedalia, 2000) but they can be inhibitory depending on their concentrations (Table 1-6). If added individually, those phenolic acids (also called hydroxycinnamic acids) can decrease *in vitro* fermentation of cellulose (Borneman et *al.*, 1986; Jung et *al.*, 1992). pCA added to wheat straw did not lead to limitation of degradation but lignin-linked pCA can potentially limit straw hydrolysis (Besle et *al.*, 1986). Borneman et *al.* (1986) observed *in vitro* that 1mM pCA is generally inhibitory for rumen bacteria studied and 10 mM FA is only inhibitory for one out of four strains. In addition, pCA was less degraded than FA in the rumen (Varel and Jung, 1986). With synthetically esterified phenolic acids to oat spelts xylan fractions, Jung et *al.* (1991 cited by Yosef and Ben-Ghedalia 2000) observed a negative correlation between concentration of esterified phenolic acids and IVDMD. In this study, esterified FA was slightly more inhibitory to IVDMD than esterified pCA (Yosef and Ben-Ghedalia, 2000). Akin et *al.* (1993a) tested the effect of esterified pCA and FA on the digestion of linked arabinoxylan by three rumen strains. They concluded that ester bonds can limit the availability of carbohydrates in a species dependent way; perhaps due to different enzymatic material especially in terms of esterases. Finally, it seems that a lot of rumen organisms have high levels of cinnamoyl esterases (responsible for hydrolysis of hydroxycinnamic acids) (McSweeney et *al.*, 1998) and thus can cut those links.

Table 1-6. Role of phenolic acids on anaerobic digestibility (*in vitro* and rumen bacteria).

Phenolic acids (concentration)	Co-substrate	Impact	References
pCA (5 mM and 10 mM)	Cellulose	Negative	(Borneman et *al.*, 1986; Jung et *al.*, 1992)
FA (5 mM)		Negative	
pCA (9 mM)	Wheat straw	Ø	(Besle et *al.*, 1986)
pCA (1 mM)	Ø	Negative	(Borneman et *al.*, 1986)
FA (10 mM)		Negative	
pCA and FA	oatspelts xylan fractions and synthetic esterification	Negative	(Jung et *al.*, 1991)

In terms of anaerobic digestion studies, FA and pCA can be converted into biogas up to 85% with acclimated microorganisms (Healy and Young, 1979). At concentration of 12 mM, FA was well converted to biogas after a lag period of fifteen days. CH_4 production of cellulose and FA mixture was slightly better than expected but a lag phase was observed (Nordmann, 2013).

1.5.2.3 Other cell-wall linkages in anaerobic digestion

Pectins bind to cellulose and are covalently and non-covalently linked to xyloglucan (Cosgrove, 2005). Pectins are principally formed by galacturonic acids that are linked together with carbohydrates and α-1,4 bounds (Sun, 2010). They constitute an amorphous structure (Le Troedec et *al.*, 2008). To a lesser extent, other molecules have a role as lignin inter-bound or lignin/hemicelluloses cross-linkage: sinapic, cinnamic, ρ-hydroxybenzoic, galacturonic and glucuronic acids (Bidlack et *al.*, 1992; Sun, 2010). Some of them (including FA and pCA) can be covalently bound to cell wall pectins (Dinis et *al.*, 2009; Sun, 2010).

Uronic acids are pretty well degraded in the rumen (Ben-Ghedalia and Miron, 1984). Monlau et *al.* (2012b) did not observe an impact of uronic acids content on methane production of various lignocellulosic residues. Thus, they may be easily digested. During anaerobic digestion, breaking bounds of pectin occurred during hydrolysis step and allowed anaerobic microorganisms to use linked fermentable carbohydrates. Pretreatment with pectinases can improve hydrolysis and subsequent methane production even if pectin is a minor compounds. Generally, activities used are high (more than 2000 U) (Pakarinen et *al.*, 2012). However, Frigon et *al.* (2012) obtained 72% methane more with only 50 U of pectinase but with an incubation of 78 days with sodium acetate.

1.5.2.4 Lignin-derived compounds

Lignin-derived compounds can bind proteins rendering them unavailable for anaerobic microbial utilization (Han et *al.*, 1975; Karunanandaa and Varga, 1996a; Velez et *al.*, 1985) and/or inactivating anaerobic digestive enzymes (Gamble et *al.*, 1996; Han et *al.*, 1975). Rumen cellulolytic bacteria can be inhibited by small phenolic compounds. The lignin compound syringaldehyde was well digested anaerobically, alone or in combination with xylose. With vanillin, even if methane was produced, some inhibitions are possible (Barakat et *al.*, 2012). Anaerobic digestion processes are pretty tolerant to phenolic compounds, depending on their concentrations (Monlau et *al.*, 2014) and their structures (Hernandez and Edyvean, 2008). Reactor mode (batch or continuous) and inoculum adaptation influence the inhibitory capacity of such molecules (Monlau et *al.*, 2014).

1.5.3 Influence of histology

1.5.3.1 Epidermis role and composition

Epidermis is composed by the outmost surface cells (Han et *al.*, 1975). Surface of plants contains, among others, silica, cutin and waxes. It plays a protective role against biotic and abiotic stress (Sun, 2010). Thus, it is difficult to degrade. In cereals, it is a non-negligible tissue: for wheat straw, epidermis represent 5 to 7% of the volume and 15% of the total mass (Zeitoun, 2011).

Silica is found in cell-wall and especially in epidermis. It provides a physical barrier to enzymatic degradation and helps to fight metal toxicity, salinity, drought and temperature stresses (Currie and Perry, 2007). In average, silica concentration in angiosperms is between 3 to 5% dry weight. It constitutes an

important part of the ash (more than 90% in wheat straw) (Han et *al.*, 2010). This small quantity is sufficient to increase mechanical resistance and to cause difficulties during pretreatment (Motte, 2013).

Cuticle is constituted by cutin. Cutin is a lipophilic polymer (fatty acids) with phenolic structures such as FA and pCA. It is embedded in waxes and found in majority on the surface of leaves and stems. Waxes are also present in the epidermis. Most plants and all gramineae are covered on aerial parts with semicrystalline wax (Sun, 2010). Epidermis (wax, cutin…) is composed by alkane, ester, alcohol, phenolic acids, fatty acids… Natural wax level in crop materials is commonly between 1% and 2%, which is sufficiently high to lower straw wettability and to inhibit bonding among particles (Han et *al.*, 2010). Lipophilic extractives constitute a small part of lignocellulosic biomass but can have an important role. They give wood its color, its odor, and, to some extent, its physical properties (Imamura, 1989). A major part of lipophilic substances are deposited on the middle lamella and on epidermal cells (cutin and waxes) to play a waterproofing role and a barrier to microbial attacks.

1.5.3.2 Anaerobic digestion at cell scale

Anaerobic degradation occurs from the center to the outer tissues (Akin et *al.*, 1974; Motte, 2013). In the center, the vacuole is easily accessible (like a pore) and outer tissues are more lignified (schlerenchyma and xylem). Outer tissues are therefore more difficult to degrade in anaerobic digestion than parenchyma (Akin, 1989; Motte, 2013).

During high solids anaerobic digestion, Motte (2013) showed that tissue kept their structure since medium lamella, silicified epidermis and cell intersections are recalcitrant. Cells from medium lamella contain very resistant lignin (Engels, 1989). One unit increase in silica content decreased forage grass digestibility by three percent (Van Soest and Jones, 1968). High content in silica can probably impede a good delignification (Zadražil, 1980).

Cuticle (lipophilic) is difficult to degrade anaerobically (Motte, 2013). Rumen bacteria split the cuticle by preferentially attacking the cell just underneath the cuticle (Akin et *al.*, 1974). Bacterial attachment is primordial to digeste a substrate. Rumenal bacteria are unable to attach to cuticle whereas anaerobic fungi hyphae are abble to penetrate the cuticle (McAllister et *al.*, 1994). If lipophilic substances of the cuticle are degraded, it could favor the accessibility to the substrate and perhaps improve methane production. Several studies showed a negative impact of lipophilic extractives on methanogenesis. Some resin acids, in particular, can inhibit anaerobic digestion at only 20 mg/L (Baroutian et *al.*, 2013).

From these findings, one may wonder on which of these parameters fungal pretreatment can act.

1.6 Qualitative effects of fungal pretreatment on anaerobic digestibility

1.6.1 Modifications of lignin: G/S (guaiacyl/synapyl) ratio

Lignin degradation is a key point to improve digestibility. Qin et *al.* (2009) showed that WRF can increase the hydrophilicity of lignin. Polar extractives, especially vanillyl compounds, were identified as products of lignin depolymerisation after basidiomycete pretreatment (Gutiérrez et *al.*, 1999).

For WRF, G units are also more difficult to degrade (Sánchez, 2009) and S units are generally depolymerized more rapidly (Akhtar et *al.*, 1997). However, some fungi like *C. subvermispora* FP 90031-sp preferentially degrade G units than S units (Akhtar et *al.*, 1997; Akin et *al.*, 1993b; Terrón et *al.*, 1995). Fungi found in coniferous wood like *Phellinus pini* seems also efficient for G units degradation (Akhtar et *al.*, 1997). The preferential degradation of S and G can be a function of fungal treatment duration; as observed by Agosin et *al.* (1985): preferential degradation for G units before 10 days and for S units after. In contrast, it was considered that S units are first erode whereas G units are attacked in a later degradation stage (Backa et *al.*, 2001). Camarero et *al.* (2001) found an influence of the MnP amount on G/S. To resume, it seems possible to increase S/G even if it is not the most frequent case. This increase is often linked to a better IVDMD: 80% improvement in the study of Akin et *al.* (1993b). Although, it is not a sufficient parameters to explain improvement since a decreasing ratio can correspond to a better digestibility (Agosin et *al.*, 1985).

1.6.2 Cellulose digestibility improvement

Concerning the crystalline cellulose, it appears that enzymes secreted by aerobic and anaerobic fungi are able to solubilize it (Sánchez, 2009; Wood et *al.*, 1988). In their review, Isroi et *al.* (2011) explained that it is possible to reduce crystallinity of cellulose with WRF. On wheat straw pretreated with *Phanerochaete chrysosporium* and with inorganic salts addition, a crystalline degree reduction from 44% to 15% was observed. It corresponded to 25% lignin loss and 27% overall sugars loss but also to 250% higher efficiency for sugar release through enzymatic hydrolysis (Zeng et *al.*, 2011). Without additives, crystalline degree can be slightly decreased (around 4% less) in pretreated substrates (Lee et *al.*, 2007; Zeng et *al.*, 2011). Yang et *al.* (2013) confirmed that fungi can open the crystalline cellulose structure and create an amorphous structure less water repellent. Yet, no improvement of crystalline index occurred perhaps because of the amorphous cellulose consumption. Müller & Trösch (1986) studied the hydrolysis by cellulase of a WRF pretreated substrate (Table 1-4). They suggested a decrease in cellulose crystallinity caused by fungi. They obtained a higher glucose yield with a longer WRF pretreatment period. Improvement of cellulose digestibility is caused by a better cellulose accessibility that can be due to a decrease in crystalline index but also to delignification (Taniguchi et *al.*, 2005).

According to Monlau et *al.* (2012b), amorphous holocelluloses loss must be limited to avoid a decrease in methane production. The availability of cellulose is very important and can be illustrated by the study of

Karunanandaa & Varga (1996a). They observed an increase in Dry Matter (DM) and Organic Matter (OM) digestibility of the treated straw in spite of a decrease in hemicelluloses and crude protein digestibility. This was probably due to a better cellulose digestibility. Similarly, Agosin et *al.* (1985) found a good IVDMD and delignification with a preferential hemicelluloses removal instead of cellulose.

1.6.3 Changes in hemicelluloses X/A (xylose/arabinose) ratio and acetate

Karunanandaa & Varga (1996b) observed a decreased X/A ratio on rice straw pretreated with *Cyathus stercoreus*. The resulting hemicelluloses were slowly digested and thus less degradable. In contrast, a rapid decrease in the arabinose content of wheat straw pretreated with *C. stercoreus* was suggested to be partly responsible for digestibility improvement (Agosin et *al.*, 1985). Thus, it is possible to improve hemicelluloses digestibility and X/A ratio with the good strain and good culture conditions. Hemicelluloses are often degraded by WRF that have endoxylanases (Isroi et *al.*, 2011). It appears that acetate can easily be degraded by WRF and even that deacetylation can sometimes occurred with a small advance prior xylose removal (Agosin et *al.*, 1985).

1.6.4 Increase of porosity

The presence of fungi can increase pore sizes and surface areas by the penetration of mycelium in SSF process (Talebnia et *al.*, 2010; Tian et *al.*, 2012). It was notably observed by scanning electron microscopy (SEM) (Isroi et *al.*, 2011). This technique even allowed Zeng et *al.* (2011) to discern more clearly cellulose microfibrils in the best pretreated samples (SSF + *P. chrysosporium* + salts) showing important lignin removal. In pretreated wood powder with *Stereum hirsutum* Lee et *al.* (2007) saw a 10-fold higher pore volume. Therefore, the accessibility to enzymatic attack is favored.

1.6.5 Actions of WRF on lignin-carbohydrates complex (LCC) linkages

Lignin 'selective' WRF are able to degrade rapidly esterified phenolic acids. Some fungi are more efficient to degrade selectively phenolic acids (Dinis et *al.*, 2009; Karunanandaa and Varga, 1996b). FA was degraded faster than pCA (Agosin et *al.*, 1985; Akin et *al.*, 1993b). It may be related to a preferential breakdown of hemicelluloses. At the beginning, FA and pCA were degraded faster than lignin, then they reached almost the same rate as lignin (Agosin et *al.*, 1985). Fungal esterases allow fungi to release FA and pCA from lignocellulosic material. To disrupt the hemicellulose-lignin association, fungal esterases act synergistically with xylanases, without lignin mineralization (Sánchez, 2009). Thanks to their esterases, anaerobic rumen fungi are more efficient than rumen bacteria to attack recalcitrant cell-wall (Akin et *al.*, 1993a). Karunanandaa & Varga (1996b) found a negative correlation between IVDMD of a fungal pretreated rice straw and pCA (r=-0.73) and cellulose/pCA ratio also (-0.69). However, it is possible that the consumption of cellulose by some tested fungi led to increased phenolic acids and or lignin concentration per cellulose unit. No correlation was found with FA.

During fungal pretreatment there is an accumulation of uronic acids. This phenomenon depends probably on fungal enzymatic material (Agosin et *al.*, 1985). Some phenolic substances (4-hydroxy cinnamic acid, FA…) sometimes implied in linkages were significantly decreased by *Phanerochaete chrysosporium* on wheat straw. They were probably digested by the fungus as nutrition source (Qin et *al.*, 2009). Even if pectins are water soluble and can be used by a lot of microorganisms (Motte et *al.*, 2014a), they seem difficult to be selectively hydrolysed by WRF. Pectin degradation by WRF were observed but simultaneously with cellulose degradation (Gamble et *al.*, 1996).

1.6.6 Release of lignocellulosic polymers main constituents

WRF decrease the degree of polymerization, creating lower molecular weight compounds (Yang et *al.*, 2013).

1.6.6.1 Lignin-derived compounds

Lignin degradation by WRF may create anti-nutritive factors for anaerobic digestion even if some compounds can be cleaved by WRF: C-C bounds breaking in condensed tannins, for example (Gamble et *al.*, 1996). However, when a significant increase of digestibility is observed for pretreated samples, this potential toxicity seems insignificant (Agosin and Odier, 1985).

Agosin & Odier (1985) studied wheat straw delignification by WRF using ^{14}C-labelled lignin. ^{14}C-polymeric soluble compounds were released. Those ones were degraded to $^{14}CO_2$ and low molecular weight ^{14}C-soluble compounds. These small compounds can be easily converted to methane and increase its production (Barakat et *al.*, 2012). After 15-20 days they were converted faster than being formed. Low molecular weight phenolics can repolymerize by the action of laccase (Sánchez, 2009) instead of entering the mycelium and being further metabolized. Degraded lignin contains a higher proportion of ionizable groups (carboxyl groups…) and this may contribute to the formation of water-soluble lignin that is very important for the improvement of *in vitro* digestibility (Agosin and Odier, 1985). Acid insoluble lignin (AIL) decreased more rapidly than acid soluble lignin (ASL) for poplar treated with *Trametes velutina* (Yang et *al.*, 2013). Since ASL is composed by low molecular weight and hydrophilic materials, this phenomenon shows lignin depolymerization and structure modifications with biopretreatment.

Finally, Müller & Trösch (1986) did not observe a good hydrolysis for a well delignified pretreated straw with *Hapalopilus rutilans*. This shows, again, that delignification is not the only parameter to take into account to improve anaerobic digestibility since hydrolysis is the limiting step. Agosin & Odier (1985) advanced that only partial delignification is necessary for achieving maximal increases in digestibility (with lignin 'selective' strains). As delignification is a secondary metabolism (Jeffries, 1987), the selective degradation of lignin is difficult to obtain.

1.6.6.2 Hemicelluloses and cellulose derived compounds

After fungal pretreatment, resulting hemicelluloses can become more recalcitrant to anaerobic digestion or IVDMD. Hemicelluloses solubilization by autoclaving followed by fungal consumption may explain this phenomenon. Lack of ammonia-N or modification of the xylose to arabinose ratio is also a possible explanation for the increased recalcitrance (Karunanandaa and Varga, 1996a; Zhao et *al.*, 2014b). Hemicelluloses loss may be less impacting than cellulose loss on IVDMD; indeed Agosin et *al.* (1985) observed good IVDMD and delignification with a preferential hemicelluloses removal instead of cellulose. Structural carbohydrates can lead to non-structural soluble carbohydrates owing to fungal metabolism. They were increased with fungal pretreatments and thus can indicate a successful fungal growth (Karunanandaa et *al.*, 1992). Conversely, Qin et *al.* (2009) found sugars losses with fungal pretreatment but anaerobic degradability was not studied. Moreover, the tested fungus (*P. chrysosporium* ME446) was perhaps non selective for lignin as observed by Karunanandaa et *al.* (1992) with other strains of *P. chrysosporium*. Released sugars increased during the fermentation of pretreated wheat straw with *Sporotrichum pulverulentum.* The degradation of cellulose and hemicelluloses was concomitant and IVDMD was not improved. Released sugars reached a maximum level then decreased with fungi that prefer hemicelluloses than cellulose and IVDMD was improved. For all tested strains, the ratio soluble sugars/soluble substances decreased during the decay of the substrate (Agosin et *al.*, 1985).

Soluble sugars concentration modifications have a role on anaerobic degradation. With fungal pretreated substrate, a raised IVDMD corresponded to higher total non-structural carbohydrates content. However, there was no correlation between IVDMD and non-structural carbohydrates content (Karunanandaa and Varga, 1996b). Monlau et *al.* (2012b) demonstrated the positive effect on anaerobic digestion of increased amounts of soluble sugars. Fungi can consume these compounds, converting them to CO_2 or fungal biomass. CO_2 transformation is a real loss for methane production but fungal biomass is digested and can produce methane.

1.6.7 Influence of histology: fungal degradation at cell scale

Penetration of the hyphae in plant cells during pretreatment provides an increased accessibility to the substrate and favors the digestibility improvement. The degradation by fungi is also function of histological composition. Some fungi can colonize all tissues (like *Cyathus stercoreus*) whereas others are localized in poor lignified areas like mesophyll (leaf parenchyma) (Karunanandaa et *al.*, 1995). The capacity to degrade cellulose can possibly influence the fungal repartition. Thanks to electron microscopy, the degradation of parenchym after a 72 h rumenal digestion was observed for a treated substrate with *Cyathus stercoreus* (Cs). Parenchym was not digested in the control sample (Karunanandaa et *al.*, 1995). With the same fungi Akin et *al.* (1995) also observed a partial degradation of sclerenchyma depending on strain and distance with the colonised end (probably caused by the action at a distance of delignifiing enzymes). In contrast to anaerobic digestion, Akin et *al.* (1995) showed that fungal attack can cause cells separation in the

parenchyma and degrade partly the middle lamella. Fungal attack was also supposed to be function of the thickness of the cell wall (Messner et *al.*, 1998).

Some fungal treatments (30 d SSF with *P. chrysosporium* or *C. stercoreus* or *P. sajor-caju*) can increase the silica content of rice straw (% TS). Thus, they are unable to remove this component (Karunanandaa et *al.*, 1995). In contrast, with a strain of *Coriolus versicolor*, a diminution of 30% in silica content was observed for a 25-days pretreated rice straw (Phutela et *al.*, 2011). It is likely that WRF can penetrate cuticle as anaerobic fungi. Gamble (Gamble et *al.*, 1996) observed cutin degradation by WRF but it was simultaneous with cellulose degradation. Several authors found a capacity for white rot fungi to degrade lNipophilic substances (Dorado et *al.*, 2001; Qin et *al.*, 2009) into fatty acids and sterols, this phenomenon could be linked to the presence of laccase (Gutiérrez et *al.*, 2006). Those components were consumed by fungi after weeks of pretreatment (Dorado et *al.*, 2001). Fatty acids take part in the anaerobic digestion reaction and sterols seem no or little toxic for anaerobic process (Vidal et *al.*, 2007). Pretreated straw surface became hydrophilic, it facilitates microorganisms attachment (Liu et *al.*, 2014). To finish, *Phanerochaete chrysosporium* on wheat straw led to a disappearance of some resin acids (Qin et *al.*, 2009). It can possibly have a good influence on anaerobic digestion process (depending on resin acids and concentrations).

To investigate biomass composition and modifications due to fungal pretreatments, measurement techniques are required, py-GC-MS is one of them.

1.7 Pyrolysis-Gaz Chromatography-Mass Spectrophotometry (Py-GC-MS): a powerful tool for structure analysis

1.7.1 Definition and interest

Several techniques exist to study lignocellulosic biomass composition like Nuclear Magnetic Resonance (NMR) or thioacidolysis. They can be very costly and required expert users (NMR) or time consuming (several hours to degrade the substrate with thioacidolysis). Pyrolysis-Gas Chromatography-Mass Spectrophotometry (Py-GC-MS) appears as a useful tool because almost no sample manipulation is required and data from different compounds are provided (carbohydrates, proteins…) (Rodriguez et *al.*, 1997). It is a degradation technique but few milligrams are necessary.

Analytical pyrolysis (pyr) was first applied in 1860 (Moldoveanu, 2001) but pyrolysis coupled to GC-MS first use occurred in 70's by the NASA to detect bacteria in liquids (Mitz, 1969). Galletti & Bocchini (1995) defined pyrolysis as the thermal fission (break) of a sample in the absence of oxygen into molecules of lower mass, low enough to be suitable for Gaz Chromatography (GC) and/or Mass Spectrophotometry (MS) and large enough to provide analytical information on the original sample. Fragments are separated and identified thanks to GC-MS. Their nature and relative distribution constitute a fingerprint characteristic of a particular sample.

Volatile compounds issued of pyrolysis are separated with GC according to their affinity with the chromatographic column used and there are identified with their mass spectra (ions abundance and mass to charge ratio). For Py-GC-MS, MS is generally equipped with an electron impact (EI) that allows breaking molecules into charged fragments, then a separation takes place in a quadrupole. The quadrupole is composed of four rods, two positively charged and two negatively charged, creating an electrical field that leads to a separation of ions according to their m/z (mass/charge) ratio.

Quantitative methods used to measure main components of lignocellulose allow good estimation but different methods give different results. This is partly due to the difficulty to isolate pure fractions (proteic residue in lignin fraction, etc.) (Reeves and Francis, 1997). Generally pyrolysis **is a semi-quantitative** method but use of internal standard is possible (Alves et *al.*, 2006). However, using a large range of lignin contents, it was possible to establish a correlation between Klason lignin and lignin estimated with pyrolysis for wood, grasses and crop residues without internal standard (Alves et *al.*, 2006; Fahmi et *al.*, 2007; Ross and Mazza, 2011). Py-GC-MS is considered as a sensitive, rapid and reproducible technique with almost no waste generation (Galletti and Bocchini, 1995).

Some compounds formed by Py-GC-MS of organic matter are known to originate from lignin, polysaccharides or also proteins, etc. (Dignac et *al.*, 2005). This technic is particularly suitable for lignin since aromatic nuclei largely retain their substitution patterns (Ralph and Hatfield, 1991). Thus, Py-GC-MS allows to differentiate lignin G-units and S-units, some authors also studied H-units but compounds derived

from H-units are often considered as unspecific, especially because they can also originate from cinnamic acids which are frequent in non-woody-plant (Camarero et *al.*, 1999; Ross and Mazza, 2011). Moreover, H-units are the less abundant units in grasses (Fahmi et *al.*, 2007) but would be the most difficult to degrade (Burlat et *al.*, 1997; Camarero et *al.*, 2001). Concerning information about carbohydrates, unique origin peaks are rare, it can be notably difficult to differentiate hemicelluloses and cellulose products (Ralph and Hatfield, 1991).

Py-GC-MS of lignocellulose was successfully **applied to study** relative amounts of lignin and carbohydrates (efficiency of isolation methods…), to classify lignin based on their S/G ratio (hybrids differentiation…), and to follow chemical changes in cell walls during plant maturation or after chemical or biological delignification. Finally, it is a useful tool to determine the origin and composition of food and feed, forest litter, compost and materials from paper industry (pulps, papers, effluents) (Galletti and Bocchini, 1995; Ohra-Aho et *al.*, 2013; Reeves and Galletti, 1993).

1.7.2 Pyrolysis products (pyrolysates)

1.7.2.1 Formation mechanisms

Samples are rapidly (few milliseconds) heated to a precisely controlled equilibrium temperature, generally in the range 600-800 °C for few seconds, and cooled rapidly in order to avoid non-isothermal pyrolysis. The same substance pyrolyzed in identical conditions gives the same degradation products (profile) that is why Py-GC-MS is a reproducible technique. Degradation of polymer is dependent on the **relative strengths of the bonds inside the molecule**. Bonds breaking form free radicals requiring rearrangement to form stable compounds that is why products reflect molecular structure, stability of free radicals, substitution and internal rearrangements. Substituents of the polymer backbone can be split off (side-group scission), the backbone can be partially cut leading to smaller polymers (chain scission) or almost entirely reverts to monomer (unzipping) (Galletti and Bocchini, 1995; Wampler, 1999). It is difficult to elaborate a general theory concerning pyrolysates formation as substrate and operating conditions interact (Moldoveanu, 2001).

Aromatic structures are generally resistant. Thereby, bonds between monomers (ether and certain C-C linkages) with lower bond dissociation energy than aromatic rings are preferentially **attacked during lignin thermal decomposition**. Breakdown products are phenols, aldehydes, ketones, acids and alcohols, generally with the retention of the original substituents (OH, OCH_3) on the phenyl ring (Simoneit, 2002). Therefore, products reflect the distribution of monomers (G- and S-units) and the types of bonds linking them (Ware, 2013). Pyrolysis of lignin forms small compounds such as H_2O, CO_2, CH_2O and CH_2O_2 and leads to the generation of free radicals, concerned molecules must then rearrange to keep a stable structure. For example, anisole can lead to benzyl alcohol formation (Figure 1-17). Different thermal reactivities exist among bonds and monolignols constituting lignin or formed with pyrolysis (easier removal of OH groups against OCH_3, etc.). A same linkage, for example ether bond (C-O-C) can be easily broken when situated

near the carbon ring (α- or β-O-4 bonds) whereas it is more resistant with a γ-carbon. C-C cleavage occurs in side chain but dehydration reactions can produce new C-C bonds increasing lignin heat resistance. After extensive oxygen removal, dehydrogenation takes place, some hydrogen atoms are even splitted from aromatic rings. Phenolic composition yield is dependent on pyrolysis temperature, aldehydes start to be reconverted at temperature higher than 600°C (C Amen-Chen et *al.*, 2001).

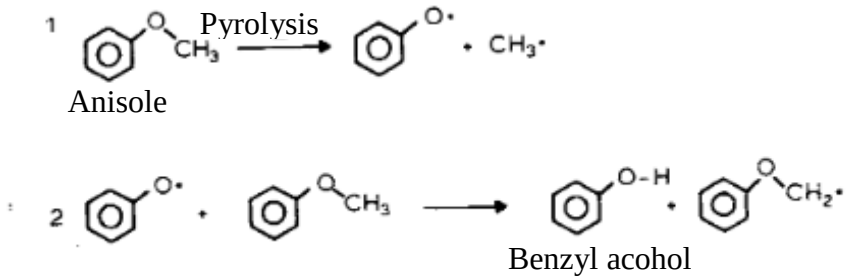


Figure 1-17. Possible rearrangements for anisole during Py-GC-MS.
(Schlosberg et *al.*, 1983)

The **depolymerization of polysaccharides** by cleavage of glycosidic bonds arises during carbohydrates pyrolysis. These processes are accompanied by dehydrations, ring opening mechanisms and cracking reactions (C-C break). Figure 1-18 illustrates some products formation mechanisms during pyrolysis of glucose polymers such as cellulose (Ware, 2013). Polysaccharides pyrolysis produces fragments of furanic and pyranic nature (rings with an oxygen replacing a carbon atom), generally deriving from sequential dehydration processes (Galletti and Bocchini, 1995) but also from fission, decarbonylation (carbonyl: R_2-C=O) and decarboxylation (carbonyl with OH) reactions (Lv and Wu, 2012). Their identification is more difficult than that of lignin markers, notably because of several isomers formation with dehydration. Isomers are difficult to differentiate on pyrograms (Galletti and Bocchini, 1995).

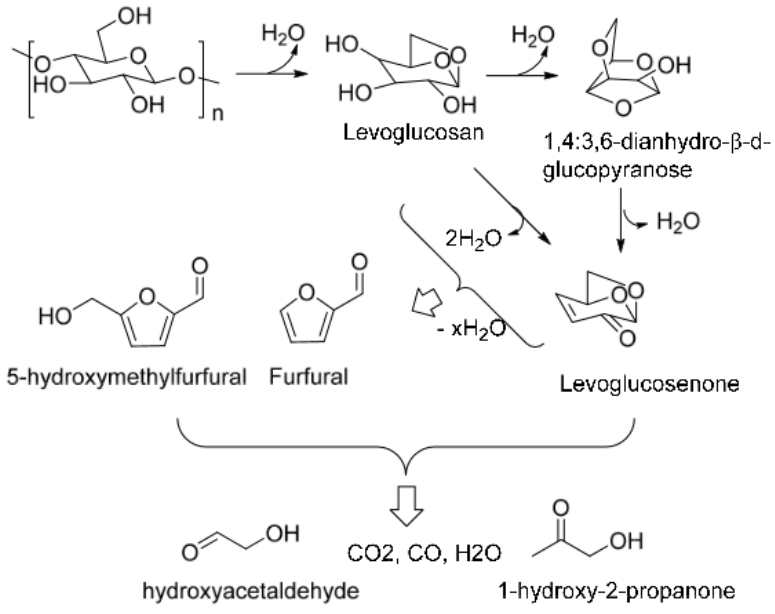


Figure 1-18. Formation of certain pyrolysates from cellulose and glucans.

1.7.2.2 Parameters influencing pyrolysates yield and nature

Temperature of the reaction is a central point because it will fix the extent of degradation: high temperature means high degradation and small products frequently nonspecific (Wampler, 1999). Lv & Wu (2012) studied thermal degradation of corn stalk and its main components (Figure 1-19). Maximum weight loss occurred in the 206-349°C range for hemicelluloses whereas it was 293-376°C temperature range for cellulose. However, solid residue (non-volatilize part) at 900°C was twice higher for hemicelluloses than for cellulose (26.4% against 13.2%). Degradation of lignin covered a wide temperature range (195–420°C) with very low mass loss rate, and generated the maximum char residue (28.9%), which can be attributed to its high carbon content. Corn stalk decomposition is function of the degradation of its three main compounds. For wheat straw thermal degradation, similar behavior occurred even if maximum degradation temperature was a bit different reflecting the difference of proportion between main polymers (Butler et *al.*, 2013).

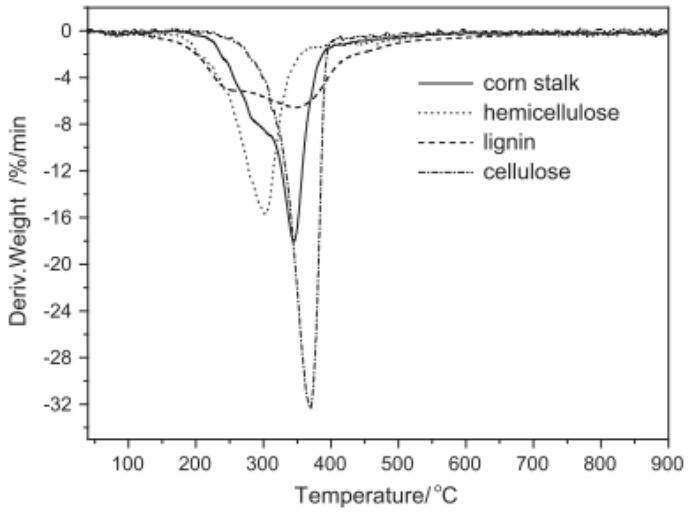


Figure 1-19. The differential thermogravimetry (DTG) curves of corn stalk and its three main components.
(Lv and Wu, 2012)

Yield and nature of pyrolysate are dependent of the process and of the sample (Figure 1-20).

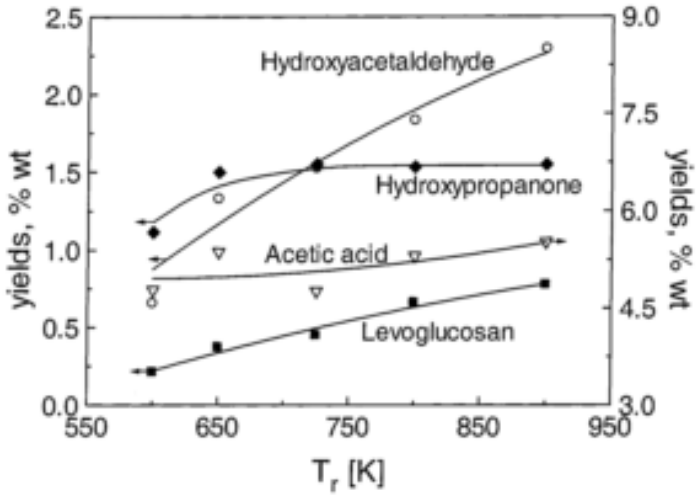


Figure 1-20. Yields of major carbohydrates, expressed as percent of the initial dry wood mass (weight total: wt), as functions of the pyrolysis temperature.
(Branca et *al.*, 2003)

Concerning process variables, influence of set point temperature was already discussed but heating rate is another important parameters. It can influence the occurrence of secondary reactions, i.e. recombination into larger fragments (C Amen-Chen et *al.*, 2001). Samples moisture, ash content (especially alkali metal content), organic composition and particle size can also modify yield of pyrolytic products (Fahmi et *al.*, 2007). To achieve the fast heating rates needed, pyrolysis zone and sample amount (less than 1 mg) must be small to favor energy transfer. Small samples avoid also temperature gradients that would hamper reproducibility owing to different pyrolysis temperatures. Finally, small samples degrade completely and rapidly and the capacity of the column is not exceeded, so good resolution is possible (Wampler, 1999). However, small sample amount limit sample representativeness especially for heterogeneous sample. The pyrolyzer should have a small internal volume to transfer the pyrolysate into the column efficiently, i. e. recovering of all products without additional reactions caused by a too long exposure to high temperature. With the same goal, a rapid inert gas carrier flow is applied. The gas is also used to purge the system: avoid oxidation during pyrolysis and protection, especially for the mass spectrometer, from intrusion of air into the system (Wampler, 1999)

Ash content (salts and metal ions) and thus also pretreatment modifying ash content (hot water or acid washing) have an influence on pyrolysates (Branca et *al.*, 2003). Some specific mineral content (potassium, sodium, magnesium and calcium) introduces catalytic effects on the degradation of biomass favoring fragmentation to low molecular weight components. Potassium concentration in plant varies over the growing season. It is considered to have the greatest influence on plant thermal conversion comparing to other metals present in biomass (Nowakowski and Jones, 2008). Washing biomass reduces the alkali metal content that is thought to inhibit the release of specific products during pyrolysis. With a washing step, cellulose derived compounds would be increased due to more depolymerisation rather than fragmentation (ring scission) leading to compounds with higher molecular weight and reducing compounds of lower molecular weight. Similar principle can be applied to lignin compounds (Fahmi et *al.*, 2007).

1.7.3 Various existing technologies for Py-GC-MS

Several heating techniques are employed in pyrolysers. Samples can directly been introduced in hot furnace (continuous mode) or placed on cold probe that will be rapidly heated to the desired temperature (pulse mode): Curie-point pyrolysis and heated filament. Curie-point temperature is reached through induction of current using a high frequency coil, when this temperature is attained (depending on the metal used), no more current can be induced. Thus, temperature in Curie-point pyrolysers is rigorously controlled. Heated filament pyrolysers have generally a platinum coil heated electronically to the chosen temperature. This system allows temperature variation and larger sample amounts (leading to better reproducibility). Moreover, sample loading is simplest as introduced in quartz tube whether than deposited on the coil as it can be the case for Curie-point heating technique (Galletti and Bocchini, 1995; Wampler, 1999). Pulse mode

with a metal coil offers maximum heating rate and heat transfer to the sample comparatively to a continuous mode with heated chamber.

Then, several GC columns are used depending on pyrolysates (and thus on sample). According Dignac et *al.* (2006), polar columns are better to separate main compounds originating from lignocellulosic biomass. The stationary phase of polar columns can be composed of polyethyleneglycol with fused silica (Figure 1-21) as for column SolGelWax, SGE. The use of semi-polar column for a good separation of carbohydrates and lignin is also reported (Fahmi et *al.*, 2007).

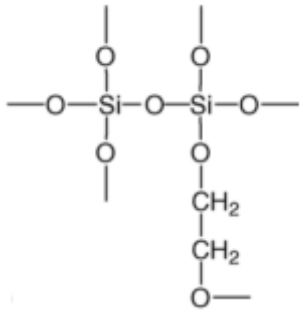


Figure 1-21. Stationary phase for column SolGelWax, SGE: polyethyleneglycol with fused silica.

1.7.4 Py-GC-MS compared to chemical methods to study lignin structure

Most applied methods to study lignin structure are nitrobenzene oxidation (NBO), CuO oxidation and thioacidolysis. They allow differentiating phenylpropanoid units (G-, S- and H- units) of lignin. Ratio of phenylpropanoid units obtained with Py-GC- MS are often compared to those obtained with usual methods. However, results are generally different between methods, especially because different bonds are cleaved. Most frequent intermonomer linkages in lignin are ether bonds (C-O-C, especially β-O-4) but some units are linked with C-C bonds.

Nitrobenzene oxidation (NBO) is a major technique for lignin structure analysis. It consists in a lignin oxidation by nitrobenzene in alkaline medium (2 M NaOH) and at high temperature (160-180°C for 2-3 hours) followed by HPLC or derivatization step and GC. NBO cleaves uncondensed bonds type aryl-ether (α-O-4, β-O-4) and α-β benzylic connections (Figure 1-4) that are alkali labile (Besle et *al.*, 1986; Lapierre, 2010). There is not really a standard protocol and interlaboratory standard deviation can be as high as 20-30% but for S/G ratio this standard deviation falls to 4-8%. S/G ratio determinated with NBO are lower than actual since carbon-carbon condensed bonds are not cleaved (5–5, β-1, β-5, β-β) and G-units are most able to form these resistant linkages than S-units (Lapierre, 2010; Monlau et *al.*, 2013). NBO is not well adapted to grasses because of the substantial amount of H-units (contrary to wood lignin) that decreases with mild alkaline treatment (Lapierre, 2010). CuO oxidation, a method similar to NBO, is better suited for grass lignin and is simpler (Camarero et *al.*, 1994; Hedges and Ertel, 1982; Pepper et *al.*, 1967), although on wheat straw results of both methods are similar. Both oxidations yield 25-75% of the treated lignin in the

form of simple phenols (Sun et *al.*, 1995). NBO and CuO oxidation allow the identification of cinnamic acids.

Thioacidolysis is an acid-catalyzed reaction for cleaving of β-O-4 bonds. Thioacidolysis is useful to study uncondensed lignin but G/S ratio obtained with this technique does not represent the whole lignin (Kishimoto et *al.*, 2010). The addition of a desulfurization step after thioacidolysis leads to the obtaining of dimers issued of condensed lignin (C-C linked units) and allows to study the proportion of the different linkages (5–5, β-1, β-5, β-β). Finally, other chemical techniques exist but are more difficult to handle like permanganate oxidation or derivatization followed by reductive cleavage (DFRC).

By taking only few compounds derived from lignin pyrolysis(rather than all lignin-derived compounds), similar results between NBO and pyrolysis S/G were obtained on Eucalyptus wood (Lima et *al.*, 2008). In presence of tetramethylammonium hydroxide, Py-GC-MS main product was issued from β-O-4 bonds cleavage that is why a linear correlation was obtained between Py-GC-MS results and thioacidolysis (Kuroda et *al.*, 2002). More studies would be required to better understand links between methods. Synthetic lignin (DeHydrogenation Polymer: DHP) with known S/G could help but existing linkages in DHP are considered to not be representative of natural lignin (Kishimoto et *al.*, 2010; Saiz-Jimenez and De Leeuw, 1986).

Another application of chemical degradation is the estimation of phenolic content. Phenolic units of lignin do not have their OH function engaged in ether bonds (Figure 1-22) and have a different reactivity than etherified units. This property allows the fixation of a methyl function on their OH function. After this methylation step, it is possible to study phenolic units with Py-GC-MS. Phenolic content of wheat straw lignin was estimated as 19% of total units. Results were similar whether alkaline CuO oxidation or thioacidolysis were employed for the determination. With Py-GC-MS, phenolic content is slightly overestimated due to the joint estimation of pyrolysis products from lignin and cinnamic acid (Camarero et *al.*, 1994).

Lignin phenolic content influences lignin solubility and high content facilitates lignin extraction (as alkaline extraction during paper pulp manufacture) (Camarero et *al.*, 1999). It is not clear if phenolic units are more susceptible to fungal degradation than etherified; as shown by Martinez-Inigo and Kurek (1997) on wheat straw alkali lignin but contrary to the study of Burlat et *al.* (1997) on wheat straw degraded with *Phlebia radiata*. Camarero et *al.* (2001) advanced that phenolic units are easier to degrade owing to a lower redox potential.

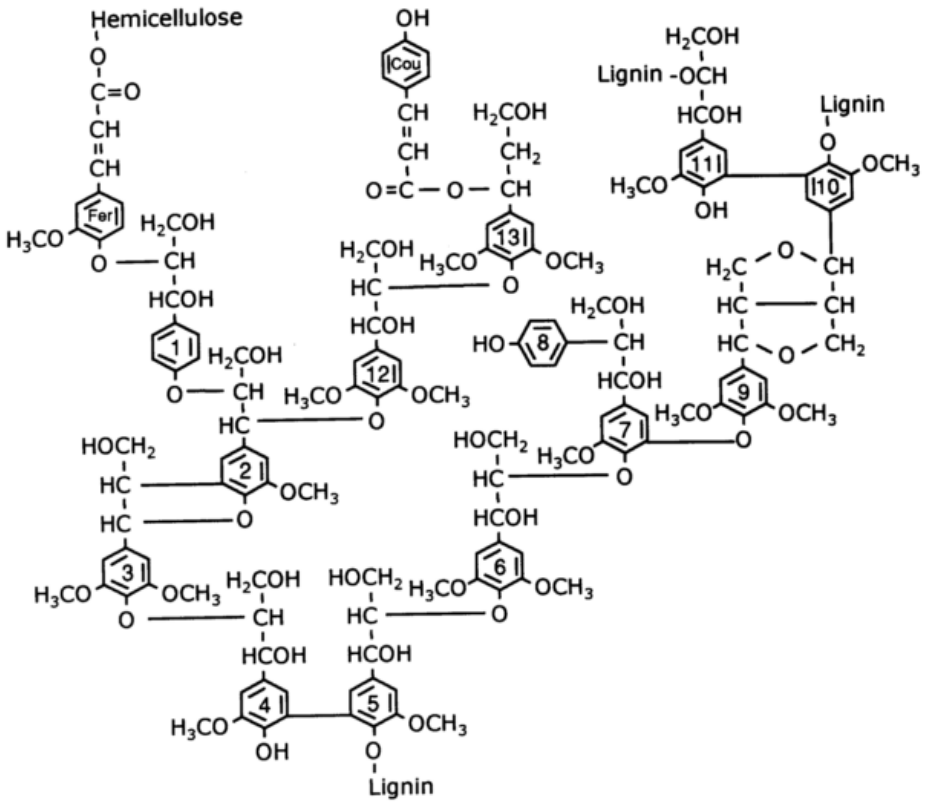


Figure 1-22. Hypothetical scheme for wheat lignin structure showing phenolic and etherified p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoids units together with p-coumaric (Cou) and ferulic (Fer) acids, the latter involved in lignin-carbohydrate linkages.

1, etherified H; 8, phenolic H; 2, 5, 7 and 10, etherified G; 4 and 11, phenolic G; 3, 6, 9, 12 and 13, etherified S.
(Camarero et *al.*, 2001)

The increased chemical agent strength to access C-C linked units goes with important risk of artifacts. Little artifacts are obtained with Py-GC-MS. Moreover, contrary to Py-GC-MS, thioacidolysis requires volatile chemicals and several preparation steps prior to chromatographic analyses (Kuroda et *al.*, 2002). However, with Py-GC-MS some structures coming from cinnamic acid (4-vinylphenol and 4-vinylguaiacol) can also derive from lignin units. This could constitute an overestimation of lignin and those compounds must not be taken into account to study lignin structure (Camarero et *al.*, 1994; del Río et *al.*, 2008). This phenomenon is often considered as a limitation of the technic even if Ralph & Hatfield (1991) estimated that differentiation of p-coumaric and ferulic acid derivatives in tissues with py-GC-MS is possible. The differentiation of phenolic and etherified lignin units is possible with Py-GC-MS if a derivatization of lignin is made before pyrolysis, e. g. a methylation step (Camarero et *al.*, 1994). Finally, grass lignins are partially acetylated and their acetylation cannot be studied with usual chemical techniques (except with derivatization followed by reductive cleavage) but it seems possible with Py-GC-MS (del Río et *al.*, 2004).

Derivatization of pyrolysates may also be done. It can improve the detectability of some analytes by modifying their behavior in the chromatographic column. It was notably applied to differentiate the origin of several carbohydrates (glucose, mannose, etc.) which is generally difficult with conventional techniques (Moldoveanu, 2001). Py-GC-MS is not only a tool to investigate lignin structure but also other components of lignocellulose.

1.8 Conclusion

White rot fungi have been widely used to improve forage digestibility for animal feed. Their use for lignocellulosic biomass treatment prior to anaerobic digestion is quite recent. Consequently, only few strains were investigated with a high diversity in pretreatments conditions (culture duration…) and it is difficult to have a clear idea about obtainable methane improvement after WRF pretreatments. Pretreatments with WRF imply complex mechanisms related to rich enzymatic systems. They include lignin and esterified phenolic acid bounds degradation but also some hemicelluloses and cellulose hydrolysis. To maximize methane production from lignocellulosic biomass, the disappearance of lignin is an important characteristic but it is not the only one. Some modifications of carbohydrates by WRF can favor digestibility: crystalline cellulose is attacked in some cases and branched hemicellulosic compounds can be removed. Nevertheless, high fermentable sugars losses must be avoided and selective delignification is required in order to limit matter losses. Thus, WRF strains should be carefully selected and the pretreatment conditions should be optimized. These conditions include water content, aeration, temperature, nutriment supplementation and pretreatment duration; as solid state fermentation processes are the most relevant for lignocellulosic biomass pretreatment.

To conclude, WRF pretreatments are very efficient to improve the biodegradability of lignocellulosic biomass. They are cheap and environmental friendly. WRF pretreatments can make new feedstock available for biogas plants; for example, woody biomass such as poplar is not suitable for anaerobic digestion but is efficiently delignified by WRF. The increase in biogas production is possible but is not systematic because organic matter losses always occur during this pretreatment. They must be taken into account to evaluate the overall process efficiency. Further research work should be carried out to help full-scale development of this pretreatment for anaerobic digestion: experiments with non-sterile conditions, studies on tolerance to contamination, pilot scale treatment experiments, etc.

Chapter 2.

Main experimental strategy

The current study investigates WRF pretreatment of wheat straw for anaerobic digestion notably because few figures on methane improvement after such a pretreatment are available for grasses and straws. The necessity to select a strain attacking lignin "selectively" was shown in the previous chapter; as well as the requirement to optimize cultures conditions. Thus, the current work began with a strain selection step followed by an optimization step. These two steps corresponded to two applied objectives of this work, their goal was the maximization of methane production. Py-GC-MS was also used to characterize pretreated straws for anaerobic digestion. The objective was to complete the answer to the central scientific question of this work: how and in what proportion do fungal pretreatments impact anaerobic digestion of wheat straw?

Anaerobic digestion was carried out using Biochemical Methane Potential tests for all those steps. Therefore, anaerobic digestion in reactor (6 L) was carried out with pretreated straw in order to be closer to full-scale anaerobic digestion plant conditions. The third applied objective consisted to study Solid State Anaerobic Digestion (SSAD) of pretreated straw.

In this chapter, common material and methods to several chapter are presented: fungal strains, BMP-tests, etc. In others chapters, specific methods and results can be found, they are divided in: selection step, optimization step, py-GC-MS study and solid state anaerobic digestion in reactor (6L). A non-exhaustive list of main analyses per goal and results chapter is presented. Then, some precisions are given about material and methods used for the general discussion (Chapter 7).

Table of content

2.1 Material and method common to all results chapters: material origin and BMP tests

2.1.1 Fungal strains

As described by Zhou et *al.* (2015a), basidiomycete strains used in this study belong to the "Centre International de Ressources Microbiennes" (CIRM-CF) dedicated to filamentous fungi of biotechnological interest (INRA, 2013) and maintained at the French National Institute of Agricultural Research (INRA; Marseille, France). The screened fungi were isolated from tropical and temperate forests.

2.1.2 Wheat straw

Winter wheat straw (*Triticum aestivum*, Haussmann) was obtained from Vivescia (Annex 2) and harvested in the North of France in 2012 (unless otherwise indicated).

2.1.3 Cultivation of *Polyporus brumalis* BRFM 985 in liquid medium

To investigate properties of fungi (BMP, py-GC-MS…), strain *P. brumalis* BRFM 985 was cultivated on 200 mL liquid medium (malt extract broth 20 g/L) in Roux flasks plugged with cotton wool. All materials and culture medium were autoclaved 20 min at 120°C. Inoculation was done with five 5-mm diameter agar disc of 7-days-old mycelia grown on MA2 (malt extract broth 20 g/L and agar 20 g/L). After 10 days at 30°C, mycelium was harvested on sterile gauze, washed extensively with ultra-pure water and freeze-dried.

2.1.4 Total Solids and Volatile Solids determination

For each substrate and for the inoculum of anaerobic digestion, TS and VS were measured according to Standard Methods (APHA, 1998). TS and VS were determined gravimetrically. TS correspond to the residue after 24h (solid samples) or 48 h (liquid samples) drying period at 105°C. It is expressed in percent of the sample initial weight. Dry residue is, then, burnt 3 h at 550°C. VS are the combusted organic matter (expressed in percent of TS or of wet weight) whereas residue of the calcination step is the mineral matter (ash). TS and VS measurements were realized in duplicates. Samples were measured again (in duplicates) when a variation of more than 1% in TS or VS content between duplicates was observed.

2.1.5 Biochemical Methane Potential (BMP) measurements: general case

BMP were measured anaerobically in batch flasks at 36 °C. The volume of each flask was 600 mL, with a working volume of 400 mL. It contained the substrate (VS substrate/VS inoculum = 1), a mesophilic inoculum from anaerobic digester stored at 37°C (≈ 3 g VS/L), 5 mL of a macro-elements solution (NH_4Cl 26.6 g/L; KH_2PO_4 10 g/L; $MgCl_2$, $6H_2O$ 6 g/L; $CaCl_2$, $2H_2O$ 3g/L), 4 mL of a microelements solution ($FeCl_2$, $4H_2O$ 2 g/L; $CoCl_2$, $6H_2O$ 0.5 g/L; MnCl2, $4H_2O$ 0.1 g/L; $NiCl_2$, $6H_2O$ 0.1 g/L; $ZnCl_2$, 0.05 g/L; H_3BO_3, 0.05 g/L; Na_2SeO_3, 0.05 g/L; $CuCl_2$, $2H_2O$ 0.04 g/L; Na_2MoO_4, $2H_2O$ 0.01 g/L), 20.8 mL of a

bicarbonate buffer (50 g/L) and water (q. s. to 400 mL). Once the flasks were prepared, a degasification step with nitrogen gas was carried out to obtain anaerobic conditions. The bottles were closed with air impermeable red butyl rubber septum-type stoppers.

Flasks were mechanically agitated. Biogas production was followed by pressure measurements (Figure 2-1) until the end of production (plateau phase). Methane volume produced between two pressure measurements is determined using the ideal gas law: $\Delta V = \frac{(y_j \times P_j - y_{j-x} \times P_{j-x}) \times V_{overhead}}{R \times T} \times \frac{R \times T_0}{P_0}$. It is, thus, necessary to known the gas overhead volume ($V_{overhead}$) and its temperature (T), as well as the pressure (P_j) in the bottle and the methane content in biogas (y_j); and finally, the methane content of the previous measurement (y_{j-x}) and the pressure after degassing of the previous measurement (P_{j-x}). The volume is expressed in Standard conditions for temperature (T_0 =273.15 K) and pressure (P_0 =1 atm), so as the ideal gas constant (R = 8.31 J/mol/K). At each measurement, endogenous production from the inoculum is calculated in the same way (in flasks without substrate) and subtracted from the global production (ΔV) measured in flasks with samples.

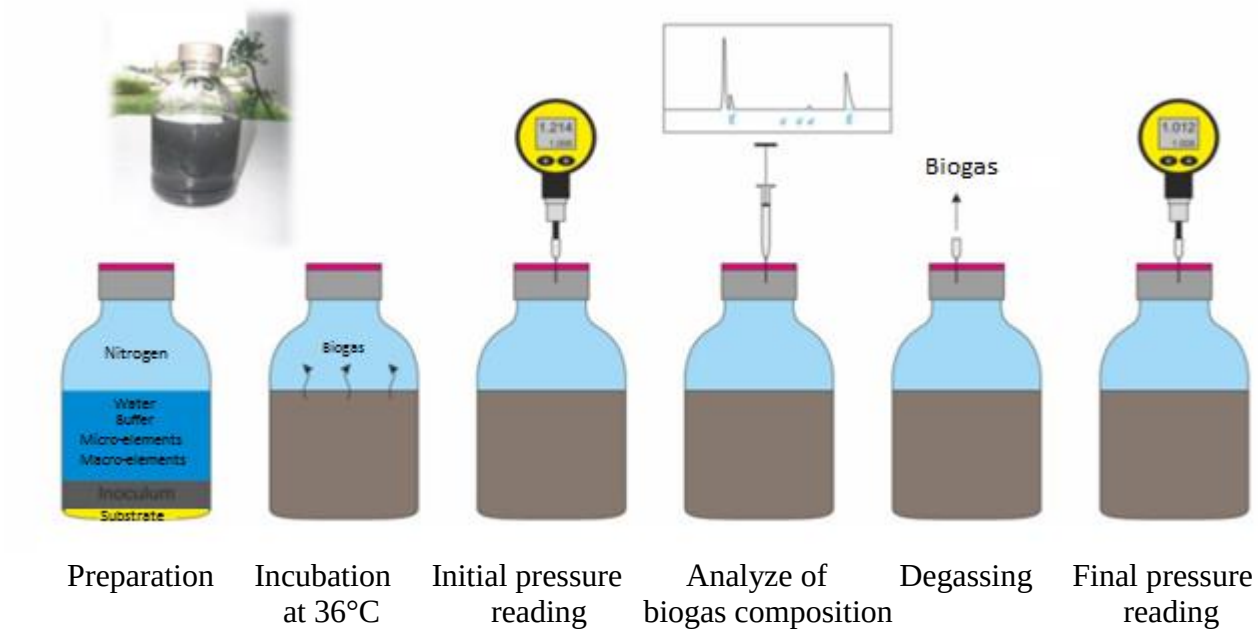


Figure 2-1. Method to follow methane production by pressure measurements.
(Adapted from Motte, 2013)

Biogas composition (notably methane content: y_j) was obtained at each pressure reading with a micro-gas chromatograph: µGC Varian lGC-CP4900. The µGC was equipped with two columns: the first one, Molsieve 5A PLOT, was set to 110°C to separate O_2, N_2, CH_4; the second one, HayeSep A, was set to 70°C to separate CO_2 and H_2S from other gases. The injector temperature was 110°C. The detection of gaseous compounds was done using a thermal conductivity detector set to 55°C. The calibration was carried out with a standard gas composed of 0.1% H_2S, 0.5% O_2, 10% N_2, 25% CO_2, and 64.4% CH4.

Measurements reliability is ensured by the sufficient production (around 350 NmL CH_4/g DCO) obtained on two positive controls containing 1 g DCO ethanol. Samples were generally measured in triplicates. Specificities occurring for BMP tests of certain samples are mentioned in corresponding results chapters. Substrate/inoculum ratio (VS basis) was the same for all BMP-tests. Even if inoculum source is constant, its activity can vary between BMP-tests (see Chapter 1, 1.1.5.1).

Biogas composition for few BMP-tests was measured with a Clarus GC 480 (PerkinElmer, USA); equipped with two columns used at 65°C: the first (RtUbond) was to separate O_2, N_2, CH_4, the second (RtMolsieve) was used to separate H_2S and CO_2 from other gases. The carrier gas was helium at pressure of 36 psi at 50 $mL.min^{-1}$. The injector and detector temperature was 200°C. The detection of gaseous compounds was done using a thermal conductivity detector. The same calibration gas than for μGC was used to calibrate the GC 480.

2.2 Overview of analysed samples

In result chapters, diverse pretreatment conditions, anaerobic digestion system and analyses were used (Table 2-1).

Table 2-1. Summary of main analyses per result chapter and scope.
Analyses in green were realized by BBF UMR; * not all samples measured.

	Chapter 3	Chapter 3 and 5		Chapter 4 and 5	Chapter 6	
	Impact of glucose	**Selection**		**Optimization**	**SSAD**	
Pretreatment system	Deep-well		Glass column		Pilot-reactor	
Number of **strains**	2	12	7	1	1	
Added **glucose** amount with starter solution (mg/g straw)	0; 50; 200; 400	50 or 200	25	0	0	
Added **diammonium** tartrate amount with starter solution (mg/g straw)	0 or 18.4	18.4 or 4.6	2.3	0	0	
Anaerobic digestion system	Ø	BMP tests			BMP tests	Batch reactors SSAD
Other measurements on substrate	Acid hydrolysis (NREL method), soluble glucose		CT **Py-GC-MS** qPCR*	qPCR*, Acid hydrolysis (NREL method), hydrolysis enzymatic, soluble glucose, **Py-GC-MS***		NTK

2.3 Material and methods specific to results overview (Chapter 7)

2.3.1 Contamination problem

2.3.1.1 Pilot-reactor cleaning verification

After cleaning and before inoculation, petri dishes were let in the pilot reactor with moisten air. Medium of culture retained were PCA (Plate Count Agar) to access "total" bacterial growth and Sabouraud with gentamicine to access fungal contaminations. PCA medium composition was: tryptone (5 g/L), yeast extract (2.5 g/L), glucose (1 g/L) and agar (12 g/L). Sabouraud (Emmons) with gentamicine medium composition was: peptone (10 g/L), glucose (20 g/L), agar (17 g/L), gentamicine (0.05 g/L). Sterile swabs moisten with sterile physiological water were used to take samples on the pilot walls and in the water tank (used to moist the air). Theses samples were also spread sterilely on PCA and Sabouraud with gentamicine. Then, petri dishes were incubated for almost 10 days at 30°C. Physiological water was preferred to mQ water to avoid osmotic lysis of microorganisms.

2.3.1.2 Wheat straw microflora

To check the absence of contaminants on wheat straw and sterile wheat straw and to test the ability of the contaminant to survive after autoclaving, wheat straw microflora was studied on autoclaved wheat straw and on contaminated straw which was autoclaved. Sterilely, 1 g straw was putted in Falcon® tube with glass beads (abrasive effect) and 9 mL of physiological water with tween 1% (to avoid spores agglutination). The medium was vortexed 2 minutes and incubated in horizontal position at 200 rpm during 45 minutes. Then, 0.5 mL was used to inoculate petri dishes with PCA and Sabouraud with gentamicine medium. Petri dishes were incubated two weeks at 30°C. This protocol was elaborated by BBF UMR.

2.3.1.3 BMP-tests

BMP-tests comparing diverse wheat straws pretreated with *P. brumalis* BRFM 985 in the pilot-reactor of the LBE. Straw without apparent fungal contaminations was the one used for SSAD (see 6.2.1). Straw considered as moderately contaminated was obtained in the same conditions, except that: particles sizes was around 10 cm for the two trays, 150 mL mQ were added before and after straw autoclaving, mix of inoculum and straw was carried out in closed bags in a cement mixer. Straw considered as highly contaminated was obtained in same conditions than the one for straw moderately contaminated but only 75 mL mQ water were added after autoclaving.

2.3.2 Enzyme assays

At the end of the pretreatment, enzymatic activities were measured by S. Zhou on water extracts (2 g TS in 40 mL mQ for 1 h at 4 °C, as for "4.2.3.4 Soluble sugars"). One unit of enzymatic activity was defined as enzymes amount necessary to release 1µmol of reducing sugars per minute (Gimbert et *al.*, 2014).

2.3.2.1 Glycoside Hydrolases (GH) activities

Enzymatic activities on carboxymethylcellulose (CMC), cellulose microcrystalline (avicel PH-101), xylans, pectins, mannan, galactomannan, arabinan and arabinogalactan were measured with dinitrosalicylique (DNS) method using glucose as standard.

2.3.2.2 Ligninolytic activities

Laccase activities were determined using ABTS (Acid 2,2'-azino-bis (3ethylbenzothiazoline-6-sulphonique) oxidation by laccases. As previously described by (Bey et *al.*, 2011), this reaction can be visualized by the reduction of DCPIP (2,6-dichlorophenol-indophenol) after zymography (electrophoretic migration on gel containing a substrate for enzymatic digestion).

Global peroxidase activity (MnP + MiP activities) was measured using 5 mM 2, 6-dimethoxyphenol in 100 mM sodium tartrate buffer (pH 5) in the presence of 0.1 mM H_2O_2 and 4 mM of sodium fluoride. Same solution without $MnSO_4$ was used to measure MiP activity. MnP activity was determined by the difference between global activity and MiP activity.

2.3.3 Wheat straw compositional analyses

2.3.3.1 Data obtained for ethanol production study and PCA

Diverse analyses were carried out on straws pretreated with several strains, most of them were already presented (e. g. TC in 3.2.2.1, fungal biomass in 5.2.3.2). Acid hydrolysis (to determine main polymers amount) and cellulose enzymatic hydrolysis were carried out by S. Zhou (as explained in 4.2.3.2 and, 4.2.3.5 respectively). Glucose released by enzymatic hydrolysis of cellulose during 72 h was notably used to calculate cellulose digestibility, as follows: $Digestibility\ (\%) = \frac{glucose\ (g)\ released\ /pretreated\ WS\ (g)}{glucose\ \ (g)\ contained\ /pretreated\ WS\ (g)}$.

Lignin, cellulose and hemicelluloses losses compared to NIC were also calculated (see 4.2.3.3). Total Kjeldahl Nitrogen (TKN) was measured in the same way as in 6.2.4.3 (Buchi 370-K distillater/titrator after mineralization with Buchi digestion unit K438). PCA was realized with SIMCA software (see 5.2.4.2).

2.3.3.2 Fourier Transform InfraRed (FTIR) spectroscopy

FTIR was used to investigate changes in functional groups for constitutive polymers of straw. Covalently bonded atoms and groups excited with infrared are set in motion. Vibrational excitation occurring is

characteristic of group component atoms. Consequently, compounds absorb infrared radiation that corresponds to their energy vibration.

FTIR spectra were obtained on a Nicolet iS10 FTIR spectrometer (Thermo-scientific) equipped with a Smart iTR module (diamond plate). Spectra were recorded in ten replicates, 16 scans with and an intervals resolution of 4 cm−1 from 4000 to 600 cm−1. Then, spectra were analyzed using the Omnic software (version 9.1).

Areas of diverse characteristic absorption bands (Table 2-2) were notably used to study relative amount of polysaccharides compared to lignin: PS1/LIG (Area at 1375 cm^{-1}/ Area at 1512 cm^{-1}) and PS2/ LIG (Area at 1158 cm^{-1} / Area at 1512cm^{-1}). Lower Order Index (LOI) was also determined using a band ratio at 1430 and 898 cm^{-1} (peaks area). Same methodology as Monlau et *al.* (2012b) was then applied to determine the amount of crystalline cellulose (%TS):

$$\text{Crystalline cellulose}_{FTIR}(\%\,TS) = \frac{LOI}{1+LOI} \times Cellulose\ NREL\ (\%\,TS).$$

Table 2-2. Assignation of absorption bands in FTIR spectra.

Absorption bands (cm^{-1})	Characteristic chemical groups	References
1512	C=C aromatic skeletal vibration in lignin	(Shafiei et *al.*, 2010; Yang et *al.*, 2009)
1430	CH_2 of crystalline cellulose	(Spiridon, 2011)
1375	C–H deformation in cellulose and hemicelluloses	(Yang et *al.*, 2009)
1158	C–O–C vibration in celluloses and hemicelluloses	(Yang et *al.*, 2009)
898	C–O–C vibration from amorphous cellulose	(Shafiei et *al.*, 2010; Spiridon, 2011)

2.3.3.3 Crystallinity measurement with X-Ray Diffractometry (XRD)

As described by Monlau et *al.* (2012b), X-ray measurements were performed by A. Barakat (IATE UMR) with a Philips Analytical X-diffractometer, using Cu Ka radiation at k = 0.1540 nm (40 kV, 40 mA). Measurements were carried out on powder compacted to small mats. XRD 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: $\text{CrI} = \frac{\text{I002}-\text{Iam}}{\text{I002}} \times 100$, where I_{002} corresponds to the counter reading at peak intensity at a 2θ 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. Crystalline cellulose was determined using the equation:

$$\text{Crystalline cellulose}_{XRD}(\%\,TS) = \text{CrI} \times Cellulose\ NREL\ (\%\,TS).$$

Chapter 3.

White-rot Fungi selection for pretreatment of lignocellulosic biomass for anaerobic digestion and impact of glucose supplementation

Objectives

- Selection of a fungal strain for pretreatment of wheat straw for anaerobic digestion
- To quantify methane improvement due to fungal pretreatments
- To understand some mechanisms implied in pretreatment: impact of glucose addition

Table of content

Highlights

- White-rot fungi pretreatment of wheat straw for anaerobic digestion
- *Polyporus brumalis* BRFM 985 most efficient strain among screened ones
- 40% more methane (CH_4) per gram of organic matter are produced
- By taking into account weight losses during the pretreatment, still 20% more CH_4
- Possible limitation of delignification with addition of nutritive solution (glucose)

3.1 Introduction

Most efficient lignocellulose degraders among fungi are Basidiomycete strains. In this phylum, Brown- and White-Rot Fungi (WRF) are able to attack lignin. Whereas Brown-Rot Fungi (BRF) attack cellulose and hemicelluloses with only small lignin modifications, WRF can lead to high lignin losses and are considered as the best delignifying organisms.

A miniaturized screening (300 mg straw per strain) with SSF allowed comparing 63 Basidiomycetes strains cultivated on wheat straw and miscanthus. They were grown on wheat straw in presence of nutrient solution (starter solution) composed mainly of glucose (200 mg/g TS straw). Then, glucose and reducing sugars released after 72 h enzymatic hydrolysis were measured for pretreated straws. Those data were analyzed with a Hierarchical Cluster Analysis (HCA) and three groups were obtained: efficient, no effect and negative effect strains. In the best case, sugar yield was up to 62% than the one of untreated control (Zhou et *al.*, 2015b).

Twelve strains were selected for further studies: eleven White-Rot Fungi (WRF) belonging to the efficient group and a Brown-Rot Fungi, *Gloeophyllum trabeum* BRFM 236 (Banque de Ressources Fongiques de Marseille). *Gloeophyllum trabeum* BRFM 236 belonged to the "no effect group" in the screening on wheat straw and miscanthus but it was the first strain for the release of reducing sugars after hydrolysis when cultivated on wheat straw (personal communication from Zhou).

Next selection step, presented in this chapter, consisted to investigate methane potential of wheat straw pretreated with preselected strains. In addition, the impact of starter solution during wheat straw pretreatment was studied.

3.2 Material and methods specific to selection step

3.2.1 Fungal pretreatment

Pretreatments were realized by S. Zhou (BBF UMR). Wheat straw composition was 37.5% of cellulose, 31.3% of hémicelluloses and 21.9% of lignin.

3.2.1.1 SSF in 24-well plates

Twelve preselected strains (Chapter 1, Table 3-1) were cultivated in three series, at different times in the year by Zhou et *al.* (2015b) in BBF laboratory. Pretreatments were carried out with two and a half 24-well plates (Whatman INC, Piscataway, USA) per strain (100 mg of straw/well). After the addition of 4 mL deionized water to each well, plates were sealed with aluminum foil by an automatic plate sealer (PlateLoc, Agilent, USA) and autoclaved at 110 °C for 30 min. After sterilization, the plates were centrifuged at 3500 rpm for 5 min, and 2 mL of the supernatant were discarded. The substrate was then washed by adding 2 mL of sterilized water to each well, stirring at 450 rpm for 10 min (Microtron, Infors AG, Switzerland) and removing 2 mL of the wash liquid after subsequent centrifuging. After three washing steps, 2 mL of nutrient

solution (starter solution) were added per well and mixed. This addition of a carbon and nitrogen source was to ensure initial fungal growth. **For the first series**, 200 mg glucose/g dry straw and 18.4 mg of tartrate diammonium/g TS were added. In contrast**, these quantities were divided by four for series 2 and 3**, notably to reduce the proportion of methane originated from the addition of the nutrient solution. The excess liquid medium (i.e. 3 mL) was removed, leading to an initial wet wheat straw weight/TS ratio equals to 11. The inoculation was carried out with one agar disc of 7-days-old mycelia (5-mm diameter) crushed and mixed with substrates using a sterilized toothpick. The plates were covered with an adhesive membrane (Breathseal, Greiner Bio-One), which allows gas exchange. The straw was then pretreated under a constant humidity during 12 days at 25°C. To allow an extensive fungal colonization, the substrates embedded in fungal mycelium were turned upside down at day 6, leaving an air space at the bottom of the well.

A control (T) was made: wheat straw treated under the same conditions but without fungal inoculation and without starter solution. Another control with starter solution was also made ($T_{starter}$). Samples were freeze-dried and weighed to determine mass losses during the pretreatment. Owing to washing steps before fungal inoculation, control straw also had mass losses (7%). Mass losses were expressed in percent of washed control straw.

3.2.1.2 SSF in columns

In Solid-State Fermentation (SSF) glass columns (20 cm × 4 cm), other pretreated samples were obtained from S. Zhou with a greater amount of substrate with seven fungi among the twelve tested in deep-well. These seven strains were the best strains of the efficient group (most different than control) after a clustering on both miscanthus and wheat straw based notably on fermentable sugars released after enzymatic hydrolysis (Zhou et *al.*, 2015b). A Brown-Rot Fungi (BRFM 236) belonging to "no effect group" was also kept by way of comparison. In the same way as previously described by Zhou et *al.* (2015a), each column contained 20 g total solids (TS) of autoclaved ground wheat straw (≈4 mm, cutting Mill SM 100, Retsch®, Germany), 25 mg of glucose/g TS and 2.5 mg of tartrate diammonium/g TS (Sigma-Aldrich, France). Each column was inoculated (Figure 3-1) with 120 mg (dry weight) ground mycelium. It was issued from liquid culture (as presented in 2.1.3) and blended with sterile deionized water at 9500 rpm for 60 s using an Ultra-turrax T25 Blender. Columns were thermostated at 28°C. Wet straw weight corresponded to 4.5 fold initial TS weight. Airflow saturated with moisture was set to 120 mL/min, measured by a ball flowmeter. For each strain, cultures in SSF columns were triplicated and the obtained pretreated wheat straws were homogenized after 21 days of pretreatment. A Non-Inoculated Control (NIC) consisting of SSF culture without inoculation was also made. Then, samples were freeze-dried.

Figure 3-1. Three steps to inoculate wheat straw for culture in SSF columns.
A. Strain conservation on agar dish. B. Realization of liquid culture in Roux flask inoculated with agar disks in static conditions. C. Sterile straw inoculated with ground mycelium before culture in SSF column.

3.2.2 Anaerobic digestion: samples characterization and normalization of BMP results

3.2.2.1 Total Carbon (TC) measurements for pretreated samples in columns

The Total Carbon (TC) was estimated by a carbon analyzer (TOC-V CSN, Shimadzu and Solid Sample Module-5000A) consisting in a combustion of the sample at 900°C with a cobalt/platinum catalyzer and pure oxygen. The carbon dioxide released during the combustion is measured by a non-dispersive infrared detector. Measurements were performed in duplicates. Glucose was used as control.

3.2.2.2 BMP-tests

BMP (see 2.1.5) of samples in deep-well were measured in duplicate, and were carried out independently for each series.

3.2.2.3 Normalization of BMP results for samples pretreated in deep-well

Because of the two different concentrations of nutrient solution and the three series of BMP measurements, results were normalized to better compare all samples. As nutrient solution implies further methane production, a correction was performed to remove the effect of the nutrient solution: theoretical amount of methane produced by nutrient solution (373 NmL/g glucose and 152 NmL/g tartrate diammonium) was subtracted from the treated straw BMP (BMP_X). Indeed, the difference between the control straw BMP (BMP_T) and the BMP of control straw with starter corresponded to the theoretical amount of methane expected for the starter (data not shown).

BMP_X were then normalized by the BMP of control straw obtained for each series (BMP_T), to ensure that results are not dependent of the inoculum that can slightly change. Finally, the improvement ratio 'Improvement VS_{pretr}' was calculated: Improvement VS_{pretr} = (BMP_X - starter)/BMP_T;

with BMP_X: BMP of pretreated straw with strain X;

starter: theoretical amount of methane produced from the nutrient solution;

BMP_T: BMP of the control straw;

BMP_X, starter and BMP_T are expressed in NmL/g of pretreated VS.

During pretreatment there were mass losses from 8 to 29% TS depending on the strain and culture conditions (see Chapter 1, Table 3-1 and Table 3-2). They can reduce the effectiveness of the pretreatment, especially if losses concern fermentable sugars (cellulose and hemicelluloses). In order to consider these losses, BMP were expressed in NmL/g of initial TS (before pretreatment) and the improvement ratio 'Improvement TS_{init}' was calculated: Improvement TS_{init} = (BMP_X - starter)/BMP_T;
in the same way as ratio 'Improvement VS_{pretr}' but with BMP_X, starter and BMP_T expressed in NmL/g initial TS and not in NmL/g VS_{pretr}, as previously.

3.2.3 Impact of nutrient solution amount

3.2.3.1 SSF in deep well

S. Grisel (from BBF UMR) carried out SSF in deep-well in the same already described conditions (12 days at 25°C) but straw was ground to 1 mm (using Retsch® SM100) for subsequent acid hydrolysis. Two strains were used (BRFM 1369 and 985), one per 24-well plate. Several glucose solutions were added before fungal inoculation: 0, 50, 200 and 400 mg glucose/g dry straw. To investigate the impact of added nitrogen, starter was also tested with the same amount previously used for series 1: 200 mg glucose/g dry straw and 18.4 mg of tartrate diammonium/g TS. Four to five wells per conditions were prepared. After fungal growth, samples were freeze-dried.

3.2.3.2 Acid hydrolysis (Klason lignin, cellulose and hemicelluloses)

The lignin, cellulose and xylan contents were analyzed following adapted NREL (National Renewable Energy Laboratory) acid hydrolysis method (Sluiter et *al.*, 2011). Two-stage sulfuric acid hydrolysis was used to determine carbohydrates (glucan and xylan). Klason lignin was the insoluble residue remaining after immersion of the samples in 72% sulfuric acid at 30°C for 1 h and then boiling the sample in 4% sulfuric acid at 120°C for 1 h. The insoluble residue was separated from the supernatant by filtration on fibreglass paper (GF-F, Whatman). Each fiberglass filter was previously dried at 105°C during 24 h in crucible and weighted. Once acid insoluble lignin recovered on filters, they were dried again in the corresponding crucible and weighted to know the dry amount of Klason lignin. The soluble fraction was used to determine the acid-soluble lignin using a spectrophotometric method. The lignin content corresponded to the summation of Klason lignin and acid-soluble lignin.

After filtering to 0.2 µm (Nylon membrane, Acrodlsc ®), the supernatant was used to determine its glucose and xylose content. A high performance liquid chromatography (HPLC) (Dionex, Ultimate 3000) equipped with a Bio-Rad Aminex HPX-87H column (heated to 35°C) and corresponding microguard were used to separate sugars monomers. A refractive index (RI) detector (Water R410) was used to detect monomeric sugars in the acid hydrolysates. The mobile phase was 0.004 M H_2SO_4 at a flow rate of 0.4 mL/min. The system was calibrated with glucose and xylose (Sigma–Aldrich) solutions at 0.2; 0.5; 1; 2 and 5 g/L. The

conversion from glucose to cellulose required a factor 0.9 (M cellulose/M glucose), glucose content was used to obtain cellulose amount, as follows:

$$Cellulose\ (\%) = \frac{[Glucose]_{(g/L)} \times V_{filtrate\ (L)}}{dry\ weight\ sample\ (mg)} \times 0.9 \times 100$$

In the same way, xylose content allowed determining xylan amount (main component of wheat straw hemicelluloses), the conversion factor used is 0.88 (instead of 0.9 for cellulose). One or two measurements were carried out, depending on sample available amount.

3.2.3.3 Determination of residual glucose

Because cellulose is calculated from glucose amount, it is necessary to estimate the remaining glucose coming from starter solution to not overestimate cellulose. As glucose is very soluble, 100 mg pretreated straw were put in 10 mL mQ heated half an hour at 50°C under agitation. This solution was then analyzed by HPLC. Owing to limited straw quantity, one sample per glucose solution and per strains was used. Residual glucose (Chapter 1, Table 3-3) was removed from total glucose obtained after acid hydrolysis to determine cellulose amount.

3.3 BMP improvement

3.3.1 Straw pretreated in 24-well plates

BMP of straws pretreated in deep-well were normalized to express ratio Improvement VS_{pretr} and Improvement TS_{init}, notably because of the use of two different amounts of nutrient solution (mainly glucose) for pretreatment (series 2 and 3 different from series 1, see 3.2.1.1 for further details).

Table 3-1 shows the improvement of methane potential relatively to the control straw and expressed per gram of pretreated straws Volatile Solids (VS): ratio 'Improvement VSpretr'. Values of this ratio higher than one show an improved biodegradability of pretreated straws. It can be observed an improvement up to 40% after pretreatments with *Trametes pavonia* and *Trametes menziesii*.

If mass losses are taken into account thanks to the ratio 'Improvement TS_{init}' (Table 3-1), up to 20% improvement was found after pretreatment with *Trametes menziesii* (BRFM 1369). Three other strains are efficient regarding to this ratio: *Trametes pavonia* (BRFM 1554), *Leiotrametes sp.* (BRFM 1048), *Ganoderma flaviporum* (BRFM 1570). Methane improvement considering mass losses is scarcely studied in literature.

From this experiment, strains that did not seem efficient with the criterion 'Improvement VS_{pretr}' were all grown with the greater amount of starter solution (series 1: 200 mg glucose/g TS straw and 18.4 mg of tartrate diammonium/g TS) which may have limited the effect of the pretreatment (as shown in 3.4). Among

series 2 and 3 (starter solution amount/4), *Trametes menziesii* (BRFM 1369) led to the best improvement even after taking into account mass losses.

Table 3-1. Performance of preselected strains for anaerobic digestion of pretreated wheat straw in deep well.

(ranged in increasing order of improvement TS_{init}).

BRFM number	Strains	Added glucose amount (*mg/g TS straw)*	Improvement VS_{pretr}	Improvement TS_{init}	BMPx /BMPT	BMPx /$BMP_{T\ starter}$	Mass losses compared to control *(% TS)*
236	*Gloeophyllum trabeum*	200	0.77 ± 0.05	0.58 ± 0.04	1.13	0.93	16.5
1161	*Ganoderma adspersum*	200	0.79 ± 0.06	0.65 ± 0.05	1.16	0.94	12.5
578	*Trametes suaveoleus*	200	0.91 ± 0.04	0.65 ± 0.03	1.28	1.04	20.5
1296	*Trametes cingulata*	200	0.84 ± 0.01	0.66 ± 0.01	1.21	0.99	15.4
985	*Polyporus brumalis*	200	0.84 ± 0.01	0.70 ± 0.01	1.21	0.98	11.0
1123	*Phlebia sp.*	200	0.85 ± 0.05	0.71 ± 0.04	1.28	1	11.6
957	*Trametes ljubarskii*	50	1.02± 0.22	0.72 ± 0.18	1.15	1	21.0
91	*Dichostereum effuscatum*	50	1.16 ± 0.07	0.95 ± 0.06	1.29	1.11	16.0
1554	*Trametes pavonia*	50	1.40 ± 0.26	0.97 ± 0.29	1.46	1.31	13.3
1048	*Leiotrametes sp.*	50	1.30± 0.07	1.05 ±0.06	1.47	1.23	17.0
1570	*Ganoderma flaviporum*	50	1.24 ± 0.22	1.10 ± 0.19	1.38	1.27	12.7
1369	*Trametes menziesii*	50	1.43 ± 0.11	1.16 ± 0.09	1.56	1.33	10.0

3.3.2 Straw pretreated in SSF columns

To complete first analyzes, other SSF cultures were carried out at a larger scale in glass columns and their BMP were measured (Table 3-2). All samples were obtained with the same small quantity of starter solution (25 mg of glucose/g TS and 2.5 mg of diammonium tartrate /g TS).

In these conditions, for all strains, even the three strains belonging to the previous series 1, straws have a higher BMP per g of pretreated VS (Table 3-2) than control straw (NIC). All pretreatments improved anaerobic digestibility from 8 to 44%. Mass losses are taken into account thanks to the BMP expressed per gram of initial TS (Table 3-2). This overall efficiency (pretreatment and anaerobic digestion) led to 21% more methane than control straw with *Polyporus brumalis* BRFM 985. This strain was the most interesting in SSF columns because it was the only one that significantly enhanced the BMP per g of initial TS. Moreover, among grasses and straws, one of the best reported results was 5% improvement for anaerobic digestion of miscanthus pretreated with *C. subvermispora* (Vasco-correa and Li, 2015).

The Brown Rot Fungi *Gloeophyllum trabeum* BRFM 236 was the nearest from control after the screening classification. As expected, BMP of straw pretreated with this strain was not very different from the one of the control. Mass losses are lower than for WRF owing likely to lower lignin modification.

Table 3-2. BMP, Total Carbon (TC), mass and carbon losses of straw pretreated in columns with different strains.
(ranged in increasing order of BMP expressed in NmL/g initial TS: BMP TSi).
[a, b] samples followed by the same letter are not significantly different and [bc] sample is different from [bd] sample (ANOVA, $\alpha = 0.05$).

BRFM number	Strains	Added glucose amount (*mg/g TS straw*)	Series number when pretreated in 24-well plates	BMP $_{TS\ final}$ (*NmL/g pretreated TS*)	BMP $_{VS}$ (*NmL/g pretreated VS*)	BMP $_{VS}$/ BMP NIC (*%*)	BMP $_{TSi}$ (*NmL/g initial TS*)	BMP $_{TSi}$/ BMP NIC (*%*)	Mass loss compared to control (*% TS*)	TC (*% TS*)	TC (*%TS initial*)	C loss (*% TS*)	Mass loss/ C loss
1369	*Trametes menziesii*	25	2	198 ± 5	210 ± 5	108	167± 6	93	20.7	44.35[b]	35.16	10.85	1.91
1554	*Trametes pavonia*	25	2	200 ± 1	212 ± 2	109	164 ± 1	92	15.2	44.34[b]	37.60	8.41	1.81
957	*Trametes ljubarskii*	25	3	249 ± 21	266 ± 23	137	172 ± 21	96	28.8	43.91[b]	31.27	14.74	1.95
NIC		25	Ø	186 ± 12	194 ± 13		179 ± 12		0	46.01[a]	46.01		
236	*G.trabeum*	25	1	207 ± 41	217 ± 43	112	184 ± 41	103	7.7	46.31[a]	42.73	3.28	2.36
1296	*Trametes cingulata*	25	1	230 ± 2	243 ± 3	125	186 ± 2	104	15.6	44.26[b]	37.35	8.67	1.80
1048	*Leiotrametes sp.*	25	2	220 ± 2	232 ± 2	120	190 ± 2	106	14.9	43.81[bc]	37.29	8.72	1.71
985	*Polyporus brumalis*	25	1	264 ± 5	280 ± 5	144	217 ± 5	121	13.7	44.93[bd]	38.80	7.22	1.89

3.3.3 Pretreatments comparison

Few studies were carried out on anaerobic digestion after fungal pretreatment. For grass and straw, the methane production can be enhanced between 4% and 63% volume per amount of pretreated TS (Chapter 1, Table 1-4, Rouches et *al.* 2016). With improvement between 2 and 44% for pretreated VS in deep well or in columns, results of the present study are of the same order of magnitude than literature. However these values do not take into account the mass loss that occurred during pretreatment. This loss can lead to a negative impact of the pretreatment as observed by Müller and Trösch (1986) on wheat straw pretreated with Pleurotus florida: 27.9% CH4 more (v/w TS) but with 42.5% TS loss for 90 days of pretreatment. In this study, impact of the pretreatment is sometimes positive even after taking into account mass losses (3% to 21%). Mass losses are very dependent of pretreatment duration. Average TS loss is normally about 26% for wheat straw degraded with WRF and it varies from 12% to 66% (Jalč, 2002) cited by (Rodrigues et *al.*, 2008). Obtained mass losses in this study are in agreement with literature for both deep well and column cultivations.

In deep well, strains that had received the greater amount of starter (series 1) were less efficient but are interesting when grown in SSF column conditions which had received a very low amount of starter solution. A negative impact of starter addition is suspected.

Taking into account standard deviation and treating separately series 1 from other samples, there are no drastic differences in strains classification according to their pretreatment ability between culture in deep-well or in column. Moreover, weight losses are of the same order of magnitude in both processes. Implied mechanisms can thus be assumed to be well represented with small straw amounts used in deep well. This technic is adapted for a screening. However, culture conditions play an important role for the effectiveness of pretreatment and must be studied to optimize pretreatment (Wan and Li, 2012).

3.4 Influence of starter solution on pretreatment

Glucose addition represents an additional cost. Moreover, glucose addition during the pretreatment seems to have a negative influence on the BMP. Several phenomena can explain the lower BMP for series 1 in deep-well: i) the correction due to the starter can be overestimated; ii) consumption of cellulose and hemicelluloses may have increased and iii) delignification may have decreased compared to samples that had received less starter.

3.4.1 Fate of the nutrient solution during the pretreatment

At the end of the pretreatment, the nutrient solution can have been converted to fungal biomass, lost under the form of CO_2 or remained on straw.

Soluble glucose remaining in samples was measured with several pretreatments conditions in deep well (Table 3-3). All the glucose from nutrient solution was consumed when added amount was 50 mg of glucose/g (as for series 2 and 3) whereas until half of the glucose remained for 200 mg of glucose/g (as for series 1).

Table 3-3. Remaining glucose for several nutrient solution amounts added for the pretreatment of wheat straw with *P. brumalis* BRFM 985 or *T. menziesii* BRFM 1369.

Strains	Added diammonium tartrate *(mg/g straw)*	Glucose added during the pretreatment *(mg/g straw)*	Soluble glucose in substrate *(mg/g straw)*
Polyporus brumalis **BRFM 985**	0	0	5.6
	0	50	3.0
	0	200	43.6
	18.4	200	12.3
	0	400	170.3
Trametes menziesii **BRFM 1369**	0	0	7.9
	0	50	4.8
	0	200	92.8
	18.4	200	112.8
	0	400	212.8

Amount of fungal biomass formed is dependent of strains and of culture conditions. Literature data transposable to this study cannot be found but by way of example, Jeffries et *al.* (1981) estimated that without limitations 2.2 mg of carbohydrates produce 1 mg of mycelial biomass for the WRF *Phanerochaete chrysosporium*. BMP of fungi are high but inferior to the one of glucose, as it is 327 ± 36 NmL/g VS for the mycelium of *P. brumalis* (BRFM 985) against 373 NmL/g VS for glucose.

Small quantities used in deep well did not allow to measure fungal biomass and it was considered that all nutrient solution remained on straw to express BMP improvement ratio. Thus, impact of starter solution was a bit overestimated and can partly explain lower results for series 1. The more glucose was added, the more fungal biomass growth can be assumed and the more the impact of starter is overestimated with ratios Improvement VS_{pretr} and TS_{init}. However, without starter correction, taking the ratio BMP_X/BMP_T (Table 3-1), series 1 seemed still less interesting than others. In addition, almost no strain of series 1 gave higher BMP than the one of control with starter ($BMPx/BMP_{NIC}$ in Table 3-1). Consequently, other reasons would explain bad results for series 1 like greater carbohydrates consumption or lower delignification.

Carbon loss under the form of CO_2 can come either from straw or from starter. To have an idea of carbon loss, Total Carbon content (TC) was measured for pretreated samples in columns (Table 3-2). Mass losses are taken into account by the expression of TC in percent of initial TS (%TS init), i.e. TS before

pretreatment. Then, C losses are expressed as the difference between TC of the control and TC (%TS init) for pretreated straw. Finally, to determine the part of mass losses due to CO_2 release: ratio mass loss/C loss was calculated. In average, this ratio reaches 1.85 for WRF, whereas it is higher for the BRF, *G. trabeum* (BRFM 236). It may reveal greater carbohydrates consumption for the BRF (Sánchez, 2009).

3.4.2 Nutrient solution and wheat straw composition

According Reid and Deschamps (1991), glucose as co-substrate with synthetic lignin can limit the delignification by WRF. With the WRF *Phanaerochaete chrysosporium*, when carbohydrates are limiting, supplementary carbohydrates cause a transient repression of ligninolytic activity (Jeffries et *al.*, 1981). First metabolism of WRF consists in mycelial growth along with easily accessible carbohydrates consumption. Then, mycelial growth stops or even decreases, and the secondary metabolism of delignification takes place in response to depletion (carbohydrates, N…) (Jeffries et *al.*, 1981; Mester et *al.*, 1996; Zadražil, 1977). Shi et *al.* (2014a) modeled this behavior for *P. chrysosporium* growing on cotton stalks (Figure 3-2). However, duration of each step is function of strain, substrate and cultures conditions.

In addition, delignification is dependent on the amount of mycelium produced during the primary metabolism. If time is sufficient for the consumption of added glucose, delignification could be enhanced due to higher fungal biomass (Jeffries et *al.*, 1981). Moreover, some strains are also able to delignify during the first metabolism (Hatakka, 1994).

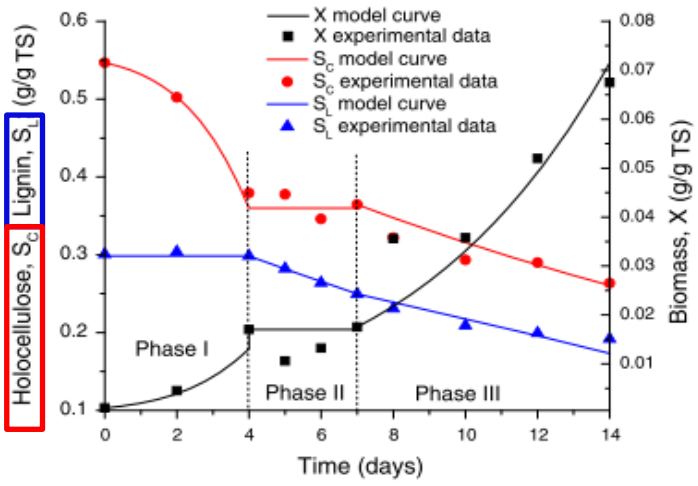


Figure 3-2. Experimental and predicted fungal biomass, holocellulose, lignin during 14 days pretreatment of cotton stalks by *Phanerochaete chrysosporium*.
(Shi et *al.*, 2014a)

As a negative impact of glucose addition was suspected during the pretreatment in deep-well and because miscellaneous parameters are identified in literature about the influence of glucose addition, two strains were cultivated with different amounts of glucose and lignin and carbohydrates were quantified in pretreated samples. The two chosen stains were: BRFM 1369 that gave good results in deep-well (series 2) and BRFM 985 that gave bad results in deep-well (series 1) and good results in column with less starter solution (see

3.2). Untreated wheat straw (UWS) was also analysed as control, measured composition for UWS is in accordance with litterature (Annex 1).

3.4.2.1 Limited delignification

Klason Lignin is the sum of Acido-Soluble Lignin (ASL) and Acido-Insoluble Lignin (AIL) but as ASL was between 0.4 and 0.6% of the pretreated material, only AIL was studied (Figure 3-3).

For each strain, AIL with addition of starter solution (glucose and N source) was superior to that with the addition of the same solution without N (Figure 3-3). For most WRF, delignification is induced under nitrogen starvation (Wan and Li, 2012), it also seems to be the case for studied strains. With a same fungus, high level of nitrogen can inhibit lignin degradation whereas low nitrogen addition could promote the degradation as observed with *Phanerochaete chrysosporium* and *Pleurotus ostreatus* (Wu et *al.*, 2005).

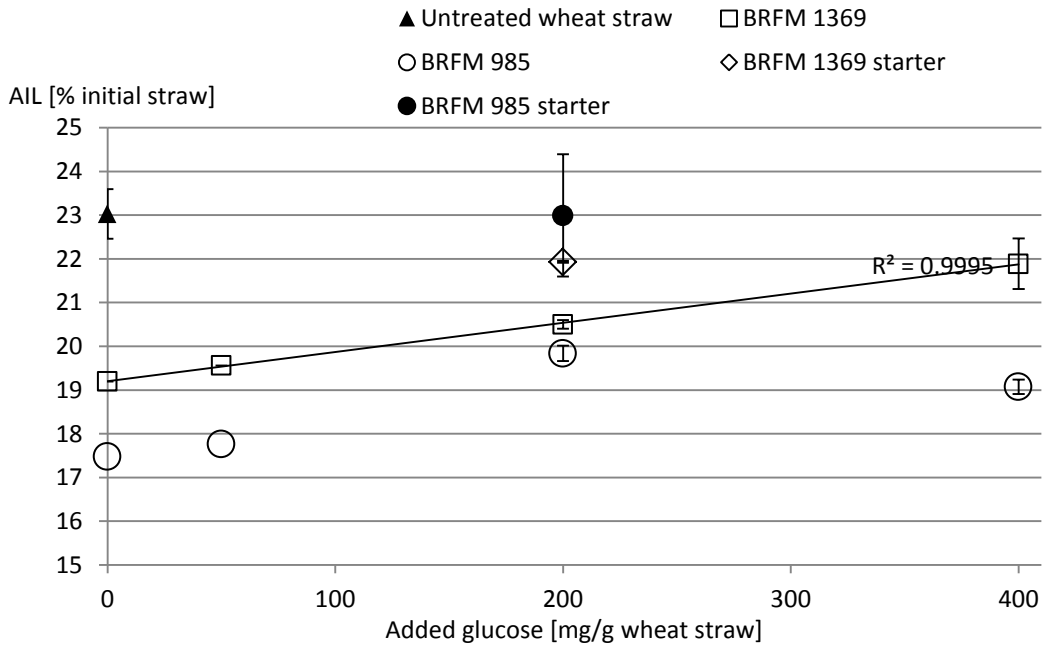


Figure 3-3. Influence of glucose addition or starter (glucose + N source) addition on delignification (Acid Insoluble Lignin content) of pretreated wheat straw with *P. brumalis* BRFM 985 and *T. menziesii* BRFM 1369.

For *T. menziesii* BRFM 1369, a clear positive correlation ($R^2 = 0.99$) exists between AIL in percent of initial TS straw and the amount of glucose added. Thus, delignification was imped because of added glucose. For *P. brumalis* BRFM 985, the correlation only has an R^2 of 0.53 but except for 400 mg glucose/g TS straw, a positive correlation seems to exist also. Several authors have observed a negative influence of glucose on Manganese Peroxidase, a key delignifying enzyme (Ha et *al.*, 2001; Mester et *al.*, 1996). Moreover, with almost ten times more glucose than ^{14}C-(RING)-lignin of poplar wood, Hatakka and Uusi-Rauva (1983) observed a slight or strong repression of the lignin degradation for four strains out of the five tested. C/N ratio in the culture medium seemed not responsible for these results. In the present study, it is estimated that glucose/lignin ratios are between 0.25/1 to 2/1. Thus, limitation of lignin degradation with glucose as co-

substrate would exist also for whole lignocellulosic biomass even with lower glucose quantity than those used by Hatakka and Uusi-Rauva (Hatakka and Uusi-Rauva, 1983).

In contrast, Wu et *al.* (2005) observed a slight improvement of delignification in pulp mill wastewaters supplemented with glucose (1 g/L glucose for 0.92 g/L lignin and 4.96 g/L COD) but with greater supplementation, repression of lignin degradation was also observed.

Those different results could be explained by the presence or absence of glucose deficiency. Glucose helps fungal growth and the more substrate is colonized the more delignification is possible. However, to take place delignification often requires carbohydrates starvation that means the consumption of glucose. Thus, if time is sufficient to reach carbohydrates starvation, efficient delignification would take place whereas with shorter time, fungi just grow thanks to added glucose (first metabolism) with almost no delignification (phase I in Figure 3-2).

3.4.2.2 Carbohydrates consumption

If few studies were carried out concerning the impact of nutrient solution on delignification, they often did not study the impact on resulting carbohydrates. N addition in nutrient solution did not influence carbohydrates consumption (Figure 3-4), as starter addition or glucose addition did not lead to significant differences in cellulose or xylan (≈ hemicelluloses) amounts with the tested concentrations.

Xylan consumption and thus hemicelluloses consumption seem independent of added glucose amount. For the two tested strains xylan content was around 14% in pretreated wheat straw whereas it was around 23% for untreated straw (Figure 3-4A).

Concerning cellulose consumption, the two strains have a different behavior. For *P. brumalis* BRFM 985, there was little or no cellulose consumption whatever the amount of glucose added (Figure 3-4B). Glucose is known to repress cellulase activity (Moreira et *al.*, 1997) but other experiments are required to determine whether it is the case for *P. brumalis* BRFM 985.

For *T. menziesii* BRFM 1369, a decrease of cellulose while increasing the amount of added glucose was observed (R^2 =0.99). Cellulases were most active for this fungi than for *P. brumalis* BRFM 985 at the end of pretreatment in SSF columns (Gimbert et *al.*, 2014) (Chapter 7, Figure 7-3). Lower delignification yield can perhaps be explained by a simultaneous consumption of glucose and cellulose leading to no C-starvation for BRFM 1369. More samples would allow to better confirmed impact of glucose addition during pretreatment on lignocellulosic composition of the substrate.

Remaining cellulose seems to be linked with remaining AIL, for both strains (Figure 3-3 and Figure 3-4): the less cellulose left, the more lignin remained. Some enzymes (glucose oxidase, etc.) are both dependent of cellulose and lignin degradation (Leonowicz et *al.*, 1999). However, further investigations would be required to draw more robust conclusions about this observation, and about influence of glucose addition during pretreatement in general.

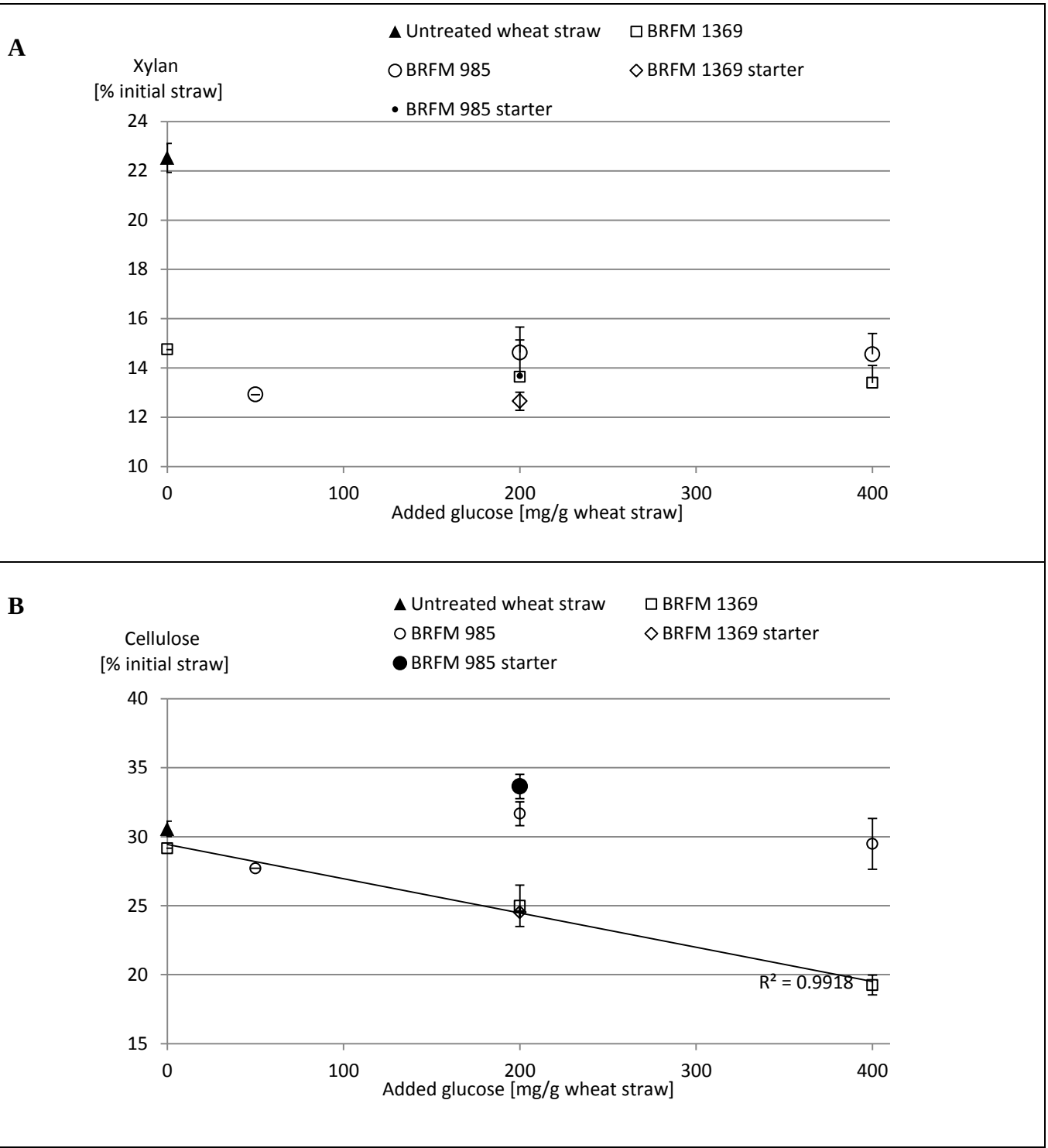


Figure 3-4. Influence of glucose or starter (glucose + N source) addition for wheat straw pretreatment with *P. brumalis* BRFM 985 or *T. menziesii* BRFM 1369.
A. On xylan content. B. On cellulose content.

To improve the BMP of pretreated substrate, it is necessary that hemicelluloses and celluloses are not too attacked concomitantly with lignin degradation. In consequence, starter addition should be limited and and *P. brumalis* BRFM 985 leading to low cellulose attack seems an interesting strain for pretreatment (Gimbert et *al.* 2014; Zhou et *al.* 2016, *under writing*). This strain was highlighted by the screening step due to its efficiency to increase fermentable sugar from wheat straw and miscanthus (Zhou et *al.*, 2015b) and lead to the best methane improvement for straw pretreated in column.

3.5 Conclusion

Some WRF are able to improve anaerobic digestion of straw up to 20%, even after taking into account mass loss. This result may be improved by pretreatment conditions optimization (duration, etc.) which will be strain-dependent. Among tested strains, *Polyporus brumalis* BRFM 985 was found to be the most efficient to pretreat straw for anaerobic digestion. However, the use of high starter amount (e.g. 200 mg glucose/g TS and 18.4 mg of tartrate diammonium/g TS) can limit fungal pretreatment efficiency. Delignification seems lower with increased glucose addition (between 50 and 400 mg/g straw) and cellulose consumption could be enhanced in a strain dependent way and for fixed culture duration.

Chapter 4.

Influence of *Polyporus brumalis* culture parameters for the fungal pretreatment: optimization step

Objectives

- To find pretreatment conditions maximizing methane production from wheat straw pretreated with *P. brumalis* BRFM 985
- To link methane production to substrate modifications occurring during the pretreatment
- To prioritize pretreatment parameters according to their impact on methane production

Table of content

Highlights

- Optimization of wheat straw pretreatment with *P. brumalis* BRFM 985 in Solid-State Fermentation glass columns
- High negative impact of lignin on cellulose hydrolysis and methane production
- Possible increased methane production rate by the rapid anaerobic degradation of fungal mycelium
- Requirement of lignin selective attack by fungi to obtain an efficient pretreatment
- Increase lignin losses and methane production thanks to metals addition during pretreatment
- Importance of pretreatment duration: minimum methane production with 12 to 15 days of pretreatment in tested culture conditions

4.1 Introduction

In the previous chapter, *P. brumalis* BRFM 985 was shown as the most interesting strains among tested ones to pretreat wheat straw for anaerobic digestion. However, fungal pretreatment efficiency is substrate and fungal strain dependent but culture conditions are also very important (Wan and Li, 2012). The study of López-Abelairas et *al.* (2013) can well illustrate this fact since they obtained 30% more cellulose digestibility and 50% more hemicelluloses digestibility with optimized conditions compared to basal conditions for wheat straw pretreatment with *I. lacteus* after 21 days.

The goals of this chapter were to find optimal *P. brumalis* BRFM 985 pretreatment conditions for anaerobic digestion, and to explain implied mechanisms. To do that, investigated culture parameters were: initial wheat straw moisture content, pretreatment temperature and duration and metals addition. Pretreatment conditions that maximized methane production of fungal pretreated wheat straw were investigated. Pretreated straws were also extensively characterized by S. Zhou and characterizations were used to explain methane production changes by structural modifications of the substrate.

4.2 Material and methods specific to optimization step

4.2.1 Fungal pretreatment for anaerobic digestion

4.2.1.1 SSF in columns

As previously described (see 3.2.1.2), pretreatments were carried out by S. Zhou (BBF UMR) in glass columns containing 20 g Total Solid (TS) of ground (4 mm) autoclaved wheat straw inoculated in sterile conditions with 120 mg TS ground mycelium. Airflow saturated with moisture was set to 120 mL/min. After pretreatment, samples (Table 4-1) were freeze-dried.

The metals solution added to some samples (1 mL) was composed of $MnSO_4$; $CuSO_4$ and $FeSO_4$ at 18 mM each and filtered sterilely at 0.2 µm. This solution is suspected to enhance fungal delignification (Salvachúa et *al.*, 2013). Diverse culture temperatures, pretreatment durations and initial moisture contents were tested according a Doehlert experimental design (Table 4-1). Four controls (NIC and NIC-M in duplicates) were also made, they corresponded to the central point (15 d, 25°C, 3.3 g wet weight/g TS) but without fungal inoculation. Among controls, two were realized with metals addition (NIC-M) and two without (NIC). Three samples (samples 23, 24 and 25) were cultivated and analyzed at a different period. Those samples were obtained at the central point of the experimental domain to estimate repeatability of the whole process (pretreatment and BMP tests).

Table 4-1. Doehlert design: coded variables for experimental points and culture parameters for controls.

Samples	Coded variables				Real variables: culture parameters			
	X1 (Metals)	X2 (Duration)	X3 (Temperature)	X4 (WW/TS i)	Metals	Duration *(d)*	Temperature *(°C)*	WW/TS initial
1	1	0.5	0	0.866	Yes	17.5	25	4.5
2	1	-0.5	0	-0.866	Yes	12.5	25	2.1
3	1	0.5	0	-0.866	Yes	17.5	25	2.1
4	1	-0.5	0	0.866	Yes	12.5	25	4.5
5	1	0.5	0.8165	0.2887	Yes	17.5	30	3.7
6	1	-0.5	-0.8165	-0.2887	Yes	12.5	20	2.9
7	1	0.5	-0.8165	-0.2887	Yes	17.5	20	2.9
8	1	0.0	-0.8165	0.5774	Yes	15	20	4.1
9	1	-0.5	0.8165	0.2887	Yes	12.5	30	3.7
10	1	0.0	0	0	Yes	15	25	3.3
11	-1	1.0	0	0	No	20	25	3.3
12	-1	-1.0	0	0	No	10	25	3.3
13	-1	0.5	0	0.866	No	17.5	25	4.5
14	-1	-0.5	0	-0.866	No	12.5	25	2.1
15	-1	0.5	-0.8165	-0.2887	No	17.5	20	2.9
16	-1	0.0	-0.8165	0.5774	No	15	20	4.1
17	-1	-0.5	0.8165	0.2887	No	12.5	30	3.7
18	-1	0.0	0.8165	-0.5774	No	15	30	2.5
NIC-1	-	-	-	-	No	15	25	3.3
NIC-2	-	-	-	-	No	15	25	3.3
NIC-M1	-	-	-	-	Yes	15	25	3.3
NIC-M2	-	-	-	-	Yes	15	25	3.3
23	-	-	-	-	Yes	15	25	3.3
24	-	-	-	-	Yes	15	25	3.3
25	-	-	-	-	Yes	15	25	3.3

4.2.1.2 BMP-tests

All samples were measured in triplicates in the same conditions (at the same time with the same inoculum), except biological replicates (samples 23, 24, 25) that were measured later with a slight different anaerobic inoculum. Caution is required to compare BMP of samples 23, 24, 25 with the one of other samples. For biological replicates (samples 23, 24, 25), inoculum concentration in BMP flasks was different (5 g VS/L) from other measured samples (3g VS/L). Moreover, their BMP tests were carried out in smaller flasks (120 mL) with a working volume of 90 mL and corresponding adapted amounts for substrates and solutions. Finally, their biogas composition was followed with Clarus GC 480 (PerkinElmer, USA).

4.2.2 Experimental design

4.2.2.1 Building of the experimental design

Response Surface Methodology (RSM) is largely used to improve processes since it enables to evaluate relationships between process variables (and their interactions) on response variables (Liyanapathirana and Shahidi, 2005).

RSM requires the definition of the experimental domain to explore using prior knowledge on the process. The present experimental design consists in four factors: pretreatment duration, culture temperature, moisture expressed in Wet Weight to initial Total Solids (WW/TS initial), and addition or not of a metals solution. Factor 'Metals' is qualitative with two levels: Yes and No (variable X_1 in the model equation). The experimental domain was characterized by its center and its variation step (Table 4-2). In the chosen domain, experimental points were defined by M. Sergent (Aix-Marseille University) and BBF team using a Doehlert uniform lattice based on coded variables called X ( ). Thus, with few experiments, the whole experimental domain was explored. Three culture temperatures, seven moisture contents and five pretreatment durations were tested (Table 4-1). A second-order polynomial model was postulated and regression coefficients calculated by the least squares method (Duménil et *al.*, 1988):

$$Y= b_0 + b_1X_l + b_2X_2 + b_3X_3 + b_4X_4 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4$$

Table 4-2. Experimental domain for quantitative variables.

Factors	Units	Center	Step
Duration (X_2)	days (d)	15	5
Temperature (X_3)	°C	25	5
WW/TS initial (X_4)	g/g	3.3	1.4

4.2.2.2 Analyze of the experimental design and response variables

R software (version 3.2.1) and the dedicated 'rsm' library were used to build response surfaces in order to optimize Biochemical Methane Potential (BMP) regarding culture parameters of *P. brumalis.* To better understand implied mechanisms, diverse other response surfaces were built: CH_4 production after 6 days and 57 days of BMP tests (lasting 6 months to reach plateau phase), dry matter and lignin losses during fungal pretreatment, water soluble sugars after fungal pretreatment and glucose yield after enzymatic hydrolysis. Regression models validity (Table 4-7) was determined using a Fischer's F-test with at least 90% confidence level comparing the variance explained by the model with the global residual variance. To ensure the robustness of RSM, global residuals took into account model residues (called residuals in Table 4-7) and the variance of the experimental response (called error in Table 4-7) for triplicated fungal pretreated samples at the central point (samples 23, 24, 25). Extrema of response surfaces inside the spherical experimental domain were calculated using models equation and Excel solver, it allowed to estimate culture parameters leading to those extrema.

4.2.3 Substrate characterization

Substrate characterizations for optimization step were carried out by S. Zhou et *al.* (2016, *under writing*).

4.2.3.1 Dry matter losses after pretreatment

After freeze-drying, samples were weighted. Dry mass losses (DML) were the difference in percent of initial straw amount (before pretreatment) and final (pretreated) amount.

4.2.3.2 Klason lignin, cellulose and hemicelluloses determination by acid hydrolysis

Cellulose, hemicelluloses and Klason lignin content of straws were analyzed in duplicates following adapted NREL two-stage acid hydrolysis method (more details in 3.2.3.2). Reducing sugars and glucose released after hydrolysis were quantified respectively using the dinitrosalicylic acid method (DNS) and the Glucose RTU kits (Biomérieux, Marcy-l'étoile, France). Hemicelluloses constituting carbohydrates were determined by difference between reducing sugars and glucose, conversion factor was 0.88 (M xylan/M xylose), as previously (see 3.2.3.2).

4.2.3.3 Lignin losses in pretreated samples compared to Non inoculated Controls (NIC)

Lignin amount in pretreated straw cannot directly be compared to lignin in NIC, Dry Mass Losses (DML) during pretreatment must be taken into account to determinate the amount of lignin residual in pretreated straw. It corresponds to the remaining lignin per amount of initial straw (before pretreatment). This amount can then be compared to lignin amount in NIC to express lignin losses for pretreated straw:

$$Residual\ lignin\ (\%) = Lignin\ (\%)\ \times \frac{(100 - \mathrm{DML})}{100}$$

$$Lignin\ losses\ (\%) = \frac{(Lignin\ (\%)\ in\ NIC - Residual\ lignin\ (\%))}{Lignin\ (\%)\ in\ NIC} \times 100$$

4.2.3.4 Soluble sugars

Soluble sugars were extracted from 2 g TS straw in 40 mL deionized water during 1 h at 4 °C and shaken at 200 rpm. Extracts were recovered by filtration through GF/F filters (Whatman) and stored at 4 °C before analysis. DNS kit was used to measure reducing sugars (Biomérieux, Marcy-l'étoile, France).

4.2.3.5 Enzymatic hydrolysis of cellulose: glucose yield

A mild alkali washing step (Salvachúa et *al.*, 2011) and enzymatic hydrolysis were performed in situ with a Tornado™ Overhead Stirring System (Radleys Discovery Technologies, United Kingdom). 6 g TS of untreated and pretreated wheat straws were subjected to alkali treatments in 100 mL of 0.1% sodium hydroxide at 50°C and 700 rpm for one hour. Then, pH was adjusted at 4.8 by addition of citrate phosphate buffer (pH 4.4, 100 mM). The suspension was further supplemented with 12 FPU/g TS substrate of commercial cellulases GC220 from *Trichoderma reesei* (Genencor Danisco, NY, USA) and 60 U/g TS

substrate of β-glucosidase from *Aspergillus niger* (Novozyme SP188). Tetracycline (150 mg/l) and cycloheximide (40 mg/L) were added to prevent any microbial contamination. The reaction was carried out at 3% (w TS/v) consistency, 50°C, and 500 rpm for 72 h. Glucose released were quantified using the Glucose RTU kit (Biomérieux, Marcy-l'étoile, France) in centrifuged samples (5000 rpm for 5 min). Glucose yield was calculated according to the following equation:

$$Glucose\ yield\ (\%) = \frac{glucose\ \ released(g)\ /((g)\ \ TS\ pretreated\ WS \times \left(1 - \text{weight loss}\ (\%)\right)}{\text{Total glucose (g)/ (g) TS Non Inoculated Controls}}$$

4.2.3.6 Real Time Quantitative Polymerase Chain Reaction (qPCR)

qPCR was used to quantify fungal biomass, according to the technic developed by Zhou et *al.* (2015a) as the suitability and the practicality of this method was demonstrated.

4.2.3.6.1 DNA isolation

Genomic DNA was extracted from fresh mycelia or SSF-treated wheat straw using the NucleoSpin® Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions except for the following modifications: samples were ground to fine powder using a cryogenic grinder (6770 Freezer/Mill). Aliquots (20–100 mg) of ground samples were transferred to 2 mL Lysing Matrix A tubes (MP Biomedicals, France) with lysis solution PL1 and processed for 30 s at 4 m/s and 20 s at 6 m/s using a FastPrep-24 instrument (MP Biomedicals). Elution of DNA from the binding matrix was performed by centrifugation after two incubation steps with 50 µL Milli-Q water at 65 °C for 10 min. DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). DNA samples were stored at−20 °C until use.

4.2.3.6.2 qPCR

Quantitative PCR was performed using qPCR Master Mix for SYBR Plus (Bio-Rad, France) on a C1000 Touch Thermal Cycler CFX96 real- time system (Bio-Rad). The oligonucleotide primers were designed to amplify a 150-bp fragment in the 5.8S conserved sequence: 5.8S-F (5′-TTTCAGCAACGGATCTCTTGGC-3′), 5.8S-R (5′-CAAACAGGCATGCTCCTCGG-3′). Reactions were performed in duplicate, in a 10 µL final reaction volume, with 2 µL of template diluted 1/100 in water, and primers 300 nM each. Contrary to Zhou et *al.* (2015a), standard curves were established by A. Rancon (BBF UMR) using serial dilutions of genomic DNA ranging from 1,8 ng/µL down to $1.8.10^{-3}$ ng/µL. The PCR cycle was as follows: 30 s at 95 °C, and then 5 s at 95 °C, and 5 s at 65 °C for 39 cycles, followed by a melt curve step (65 to 95 °C, with 0.5 °C steps). The melting curve (fluorescence=f°(Temperature)) was observed to check the presence of a single amplicon. A shoulder on this curve shows the co-existence of double-stranded and single-stranded configurations and thus two different amplicons (with different temperature of dissociation). All qPCR runs included a negative control without DNA template. Number of quantification cycles were determined using the regression mode of the Bio-Rad CFX Manager™ software (v 3.0).

4.3 Relationships between anaerobic digestion and substrate characteristics

4.3.1 Methane production results during BMP tests

BMP values are presented in Table 4-3. As expected, it can be noticed that BMP (NmL/g VS pretreated) of non-inoculated controls (NIC) was identical whether they received metals solution (NIC-M) or not during the pretreatment. BMP of NIC is superior to BMP of untreated straw (≈227 NmL/g VS against 200 NmL/g VS). Similar observation was already reported with yard trimmings and was attributed to the solubilization of hemicelluloses by autoclaving (Zhao et *al.*, 2014b). However this explanation is unlikely in the current study since with 1 mm wheat straw, the same BMP was obtained comparing raw and autoclaved substrates (Table 4-4). The consumption of recalcitrant matters (lignin, crystalline cellulose…) probably did not occur for NIC since they were in sterile conditions but sugars solubilization during the stay in glass columns seems possible and would have raised BMP. Indeed, soluble sugars content is an important factor for the enhancement of BMP values (Monlau et *al.*, 2012b).

Taking into account standard deviations, very few pretreated samples had a BMP (NmL/g VS pretreated) superior to culture control (NIC) (Table 4-3), namely samples 5 and 9. In the best case (sample 5), BMP (NmL/g VS) was increased by almost 20%. Those samples were obtained with the same culture conditions (30°C, 3.7 g WW/TS i, with metals addition) except for incubation time that was 17.5 days for sample 5 against 12.5 days for sample 9. BMP of pretreated straw can be decreased comparing to controls because of holocelluloses consumption by *P. brumalis* BRFM 985. Holocelluloses especially amorphous structures, constitute the substrate of AD (Monlau et *al.*, 2012b). For this reason, culture conditions of *P. brumalis* BRFM 985 must be optimized in order to delignify selectively the substrate and thus to obtain an efficient pretreatment. However, as there are mass losses during pretreatment, they must be taken into account to evaluate the global efficiency of pretreatment. Thus, BMP were expressed per gram of initial matter (matter before pretreatment).

Taking into account mass losses (Table 4-3) and standard deviation, no fungal culture conditions allow to improve BMP (NmL/g TS i) compared to controls. In contrast, 21% more methane (NmL/g TS i) were obtained from wheat straw pretreated with the same strain (Chapter 1) in other culture conditions (21 days, slight starter solution addition…).

Methane yield after 6 days of BMP tests was studied to better understand mechanisms implied in BMP modifications. A rapid start-up is linked to easily accessible compounds (soluble sugars and proteins…). Methane yield after 57 days of BMP tests was also studied. BMP tests cannot be directly used to predict full-scale plant performances (Carlsson et *al.*, 2012) but two months duration seems adapted to digest straw in AD plants. Pretreatment optimization for methane production after 57 days of BMP tests would be more relevant from an industrial point of view than optimization for BMP values (obtained after ≈ 6 months).

Table 4-3. Methane production and characterizations of pretreated and control straws.

minimum and **maximum** values among pretreated samples. *measured by S. Zhou.

Samples	BMP (NmL/g TS i)	BMP (NmL/g TS pretreated)	BMP (NmL/g VS pretreated)	CH_4 production at day 6 (NmL/g VS pretreated)	CH_4 production at day 57 (NmL/g VS pretreated)	CH_4 production at day 6 (NmL/g TS i)	CH_4 production at day 57 (NmL/g TS i)	Dry Mass Losses* (%)	Water soluble carbohydrates* (mg/g TS)	Glucose Yield* (%)	Lignin losses* (%)	Lignin* (%)
1	188 ± 10	232 ± 13	247 ± 14	90 ± 16	214 ± 10	71 ± 12	164 ± 7	18.9 ± 1.3	41 ± 1	**35.6** ± 0.7	28.7 ± 0.7	17.6 ± 0.3
2	151 ± 3	173 ± 3	183 ± 3	48 ± 10	155 ± 17	41 ± 8	129 ± 14	12.9 ± 1.3	22 ± 0	13.8 ± 1.0	13.1 ± 0.6	22.5 ± 0.1
3	181 ± 24	221 ±29	235 ± 31	68 ± 2	187 ± 9	54 ± 1	144 ± 7	18 ± 0.1	36 ± 0	19.0 ± 1.2	19.3 ± 0.4	21.6 ± 0.1
4	193 ± 14	229 ± 17	243 ± 18	86 ± 4	211 ± 14	71 ± 4	168 ± 11	15.4 ± 0.8	33 ± 1	26.6 ± 0.6	22.2 ± 0.4	20.0 ± 0.1
5	190 ± 3	253 ± 4	**272 ± 4**	114 ± 3	248 ± 7	**82** ± 2	173 ± 5	**25.0 ± 0.4**	**84 ± 3**	**36.4** ± 0.4	**32.7** ± 0.2	17.5 ± 0.1
6	147 ± 25	*154 ± 26*	163 ± 28	54 ± 5	139 ± 26	50 ± 5	125 ± 23	*5.1 ± 1.2*	12 ± 1	14.1 ± 0.4	*1.4* ± 1.0	23.3 ± 0.1
7	186 ± 53	212 ± 61	225 ± 65	56 ± 11	183 ± 29	48 ± 9	151 ± 24	12.4 ± 1.4	20 ± 1	12.0 ± 0.2	12.8 ± 1.0	22.1± 0.5
8	143 ± 14	157 ± 16	167 ± 17	56 ± 1	138 ± 2	49 ± 1	118 ± 2	9.1 ± 0.6	20 ± 1	12.3 ± 0.4	9.9 ± 0.6	22.4± 0.1
9	**206 ± 12**	**251 ±15**	268 ± 16	94 ± 4	230 ± 20	74 ± 3	**176** ± 16	18.2 ± 0.5	31 ± 2	31.0 ± 0.6	24.6 ± 0.4	19.0 ± 0.2
10	175 ± 32	228 ± 41	244 ± 44	94 ± 7	212 ± 36	69 ± 5	152 ± 26	23.1 ± 0.4	40 ± 2	22.1 ± 1.2	26.7 ± 2.5	20.1± 0.3
11	159 ± 40	210 ± 53	226 ± 57	77 ± 10	198 ± 52	56 ± 7	140 ± 37	24.2 ± 0.5	38 ± 1	16.7 ± 0.4	23.8 ± 0.3	22.0 ± 0.1
12	185 ± 49	212 ± 56	224 ± 60	74 ± 6	184 ± 11	48 ± 4	152 ± 25	12.6 ± 1.1	18 ± 0	14.4 ± 0.3	11.5 ± 0.2	22.9 ± 0.1
13	154 ± 13	193 ± 16	205 ± 17	96 ± 5	172 ± 8	58 ± 4	129 ± 6	20.0± 0.9	28 ± 2	24.4 ± 0.4	24.4 ± 0.5	20.0 ± 0.2
14	145 ± 26	163 ± 29	174 ± 31	59 ± 4	135 ± 15	*39* ± 4	112 ± 12	11.1 ± 0.3	20 ± 5	*7.9* ± 0.2	8.3 ± 1.2	24.0 ± 0.1
15	152 ± 10	165 ± 11	176 ± 12	72 ± 1	150 ± 9	51 ± 1	129 ± 8	8.1 ± 1.0	*11 ± 1*	11.9 ± 0.4	10.2 ± 1.4	22.5± 0.7
16	143 ± 10	159 ± 11	170 ± 12	70 ± 5	142 ± 10	47 ± 4	120 ± 8	10.2 ± 0.5	16 ± 0	10.0 ± 0.2	8.4 ± 0.5	23.0 ± 0.2
17	145 ± 9	181 ± 12	194 ± 13	96 ± 3	168 ± 6	59 ± 2	125 ± 4	19.9 ± 0.7	23 ± 3	29.8 ± 0.5	20.0 ± 0.4	21.8 ± 0.1
18	*131 ± 13*	165 ± 16	178 ± 18	87 ± 7	150 ± 14	54 ± 5	*111* ± 10	20.4 ± 0.6	20 ± 2	9.4 ± 0.4	20.6 ± 1.8	23.0± 0.4
NIC-1	204 ± 6	206 ± 6	217 ± 6	_	_	_	_	1.3 ± 0.8	8 ± 1	26.5 ± 0.5	0	23.1 ± 0.2
NIC-2	210 ± 7	216 ± 8	227 ± 8	_	_	_	_	2.6 ± 0.2	7 ± 1	27.2 ± 0.7	0	22.9 ± 0.8
NIC-M1	214 ± 14	216 ± 14	227 ± 15	_	_	_	_	0.7 ± 0.7	7 ± 1	27.5 ± 1.1	0	23.0 ± 0.1
NIC-M2	220 ± 6	216 ± 8	227 ± 8	_	_	_	_	1.7 ± 1.5	10 ± 1	27.6 ± 0.3	0	23.2 ± 0.1
23	327 ± 8	_	_	_	_	93 ± 5	242 ± 9		0	21.9 ± 0.7	22.8 ± 0.2	20.7 ± 0.1
24	292 ± 24	_	_	_	_	90 ± 9	222 ± 15		0	22.9 ± 0.7	24.1 ± 0.7	20.4 ± 0.1
25	265 ± 12	_	_	_	_	88 ± 12	208 ± 28		0	24.5 ± 0.7	24.6 ± 0.3	20.3 ± 0.1

Table 4-4. Absence of influence of autoclaving and freeze drying on BMP (NmL/g VS) of wheat straw.

Wheat straw harvested in 2013 (1 mm)	Autoclaved	213 ± 16
	Raw straw	229 ± 2
Wheat straw harvested in 2012 (1 mm)	Autoclaved and freeze-dried	238 ± 2
	Raw straw	238 ± 9

4.3.2 Parameters influencing anaerobic degradability

Monlau et *al.* (2012b) showed the negative impact of lignin and crystalline cellulose content and the positive impact of soluble sugars, protein and amorphous holocelluloses on the BMP of lignocellulosic biomasses. A mathematical model was built to predict BMP: centered and reduced coefficients were close except for lignin (Figure 4-1). The fungal pretreatment can influence the BMP value by a delignifying action (positive impact). Holocelluloses consumption by fungi must be minimized since they are the substrate of anaerobic digestion.

BMP (NmL CH_4 g/g TS) = 303.14 – 4.53*Lig (% TS) + 0.77*SolSu (% TS) + 1.28*Pro (% TS) -1.59*Cri (% TS) + 0.61Am (% TS) +1.33Ua (% TS)

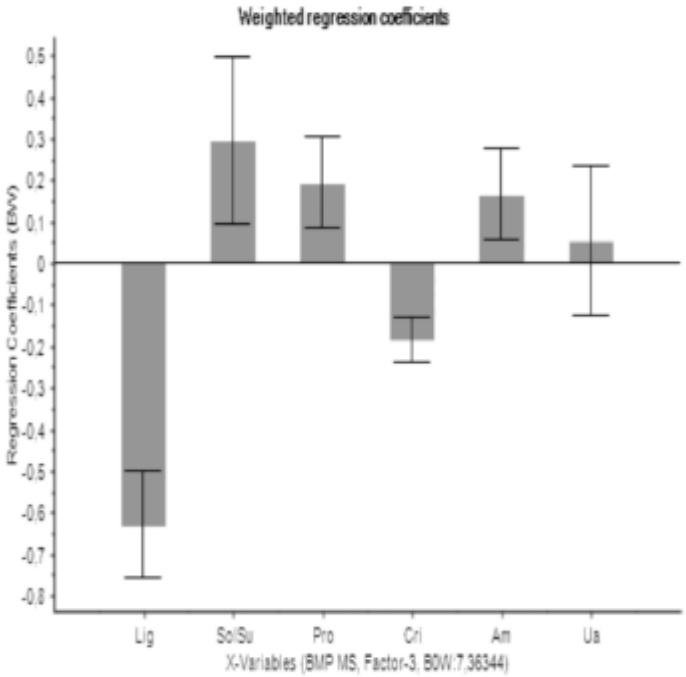


Figure 4-1. Centered and reduced regression coefficients with their 95% confidence intervals for the prediction of methane potentials and predictive equation.
Lig: Lignin. SolSu: Soluble Sugars. Pro: Protein. Cri: Crystalline cellulose. Am: Amorphous holocelluloses. Ua: Uronic acids. (Monlau et *al.*, 2012b)

Some composition analyses of pretreated straws were carried out by Zhou (BBF project partner, INRA of Marseille). Cellulose crystallinity was not studied but enzymatic hydrolyses were achieved. Glucose amounts released by enzymatic hydrolysis are dependent of lignin amount and cellulose crystallinity since they must be accessible. Crystalline cellulose represents around 50% of cellulose in wheat straw (Monlau et *al.*, 2013) (Annex 1). According literature review (Chapter 1, 1.6.2), lignin selective WRF do not preferentially consume amorphous cellulose and consequently do not increase cellulose crystallinity. Slight decrease in cellulose crystallinity can even be observed. Concerning protein content that is a minor compound in wheat straw ≈ 3% (Lee, 1997), it can be increased by the presence of fungi that have poor N requirements. Li et *al.* (2001) reported an increase of 6.2% on cottonseed hull where WRF *Pleurotus*

ostreatus were cultivated. WRF are rich in proteins and carbohydrates, e. g. *Pleurotus ostreatus* has a protein content of 24.3%TS and contains 13.4%TS of glycogen (Hadar and Cohen-arazi, 1986).

4.3.3 Negative impact of lignin amount on methane production

Negative correlation between lignin content and anaerobic degradability is known (Chandler and Jewell, 1980; Monlau et *al.*, 2012b). Even after only 6 days of methane production (NmL/g VS $_{pretreated}$), the negative impact of lignin was visible (Figure 4-2).

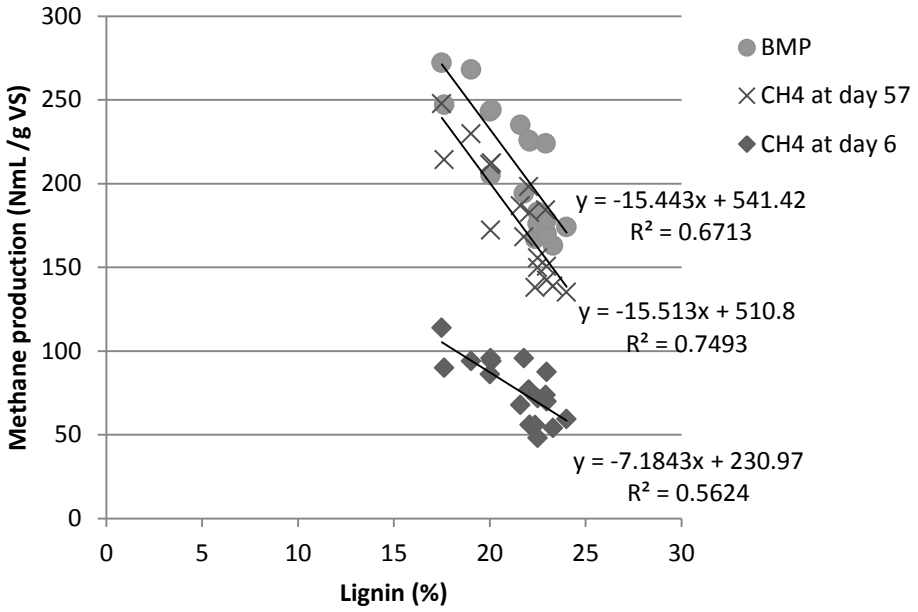


Figure 4-2. Significant negative correlations between lignin amount and methane production whatever the duration of anaerobic digestion.

Lignin influence less the CH_4 production at day 6 than at day 57 because easy accessible compounds are consumed in first days of reaction. If the correlation is a bit weaker for BMP rather than for CH_4 production at day 57 it would be due to the digestion of some recalcitrant compounds like soluble lignin fractions and slight lignin amounts. Small digested proportions of those compounds would increase with anaerobic digestion duration (Benner and Hodson, 1985; Motte et *al.*, 2015; Sleat and Robinson, 1984; Somayaji and Khanna, 1994). Lignocellulosic composition analyses on solid degraded straw residues during and after BMP tests would help to conclude on this point.

Response Surfaces (RS) were built for lignin losses during pretreatment (Annex 3 and Annex 5). As expected, lignin losses were higher with metal addition (Table 4-6 and Annex 3). Metals solutions were composed of Fe^{2+}, Mn^{2+} and Cu^{2+} that are constituents or co-factors of WRF ligninolytic enzymes (Tian et *al.*, 2012). They can favor not only ligninolytic activity but also the non-enzymatic degradation of lignin (Fenton reaction) (Salvachúa et *al.*, 2013). Lignin losses are maximized with high culture duration, sufficient water amount (≈3.8) and high temperatures (Table 4-6).

4.3.4 Influence of fungal mycelium on CH_4 production

The good anaerobic degradability of *P. brumalis* BRFM 985 freeze-dried mycelium was evocated in the previous chapter (327 ± 36 NmL/g VS). The BMP of fungal mycelium was 127 NmL/g VS more elevated than the one of untreated straw (200 ± 6 NmL/g VS). Moreover, the AD of fungal biomass is quite rapid. In Table 4-5, it can be observed that methane production from fungal biomass reached 93.9% of the BMP value in 33 days whereas, at the same date, average value for samples pretreated with metals addition reached only 73.7% of the average BMP value. In Table 4-5, it can also be found a comparison between the most degradable sample (sample 5) and the least degradable one (sample 8) among samples pretreated with metal addition. Moreover in Figure 4-3, it can be seen that the anaerobic digestion start-up for fungal mycelium was faster than for straws. During the BMP test, methane production began on day 1 for straws whereas it started directly after anaerobic inoculation for the mycelium of *P. brumalis* BRFM 985. After 6 days of production, methane yield was almost twice higher for mycelium than for the average of straws pretreated with metals.

Table 4-5. Methane production during the BMP test expressed in NmL/g VS and in percentage of the BMP value.

Days	BRFM 985	Sample 5	Sample 8	Average for samples with metals
6	139 ± 10 (42.4%)	114 ± 3 (41.9%)	56 ± 1 (33.5%)	76 ± 6 (33.3%)
33	308 ± 34 (93.9%)	228 ± 5 (83.8%)	128 ± 2 (76.6%)	168 ± 15 (73.7%)
57	320 ± 37 (97.6%)	248 ± 7 (91.2%)	138 ± 2 (82.6%)	192 ± 17 (84.2%)
BMP	328 ± 36	272 ± 4	167 ± 16	228 ± 20

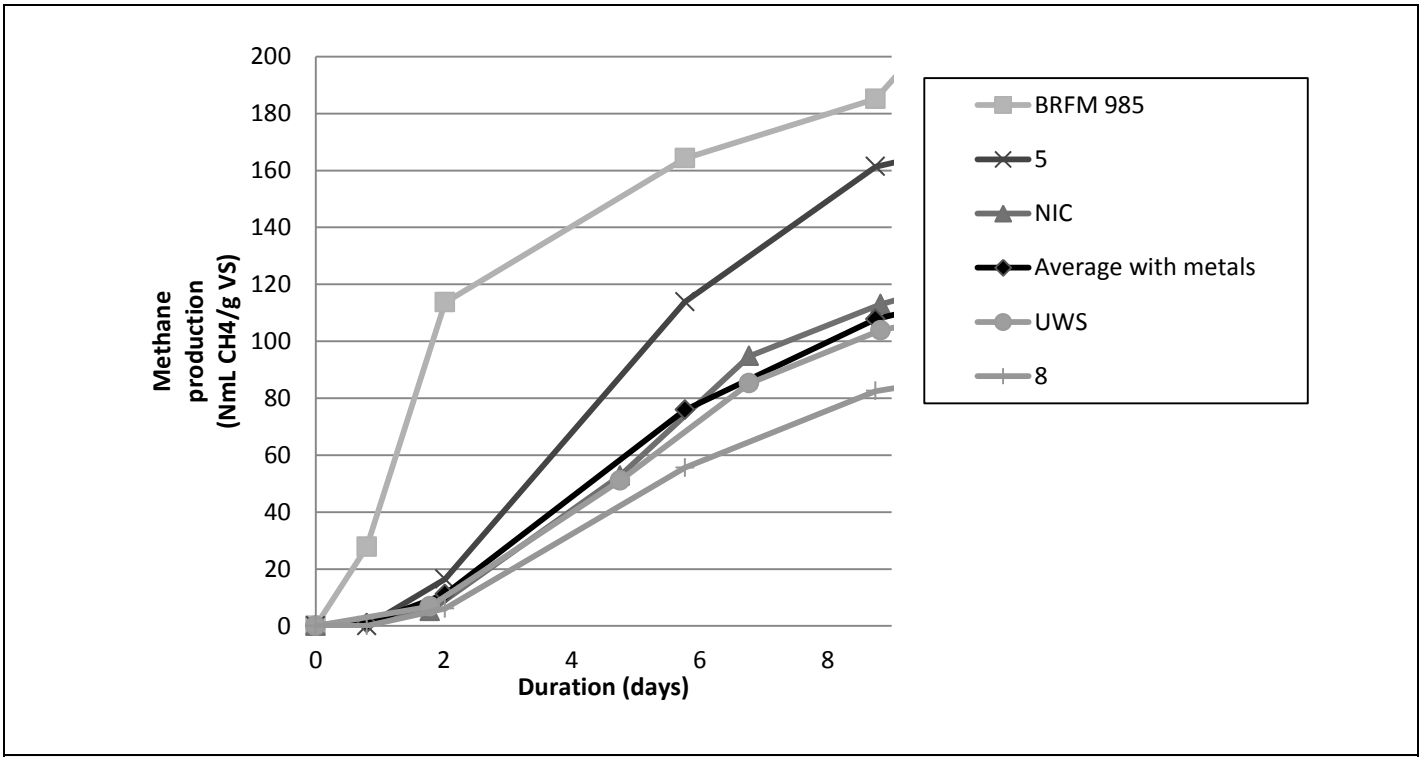


Figure 4-3. Start-up phase of BMP-tests for *P. brumalis* BRFM985 mycelium, pretreated straws with metals addition, non inoculated control with metals addition (NIC-M2) and untreated wheat straw (UWS).

Even without substrate modifications, the mere presence of the mycelium on straw would increase the substrate BMP. To investigate the influence of fungal mycelium on methane yield of pretreated straws, qPCR analyses were carried out on few samples pretreated with metals addition (Figure 4-4).

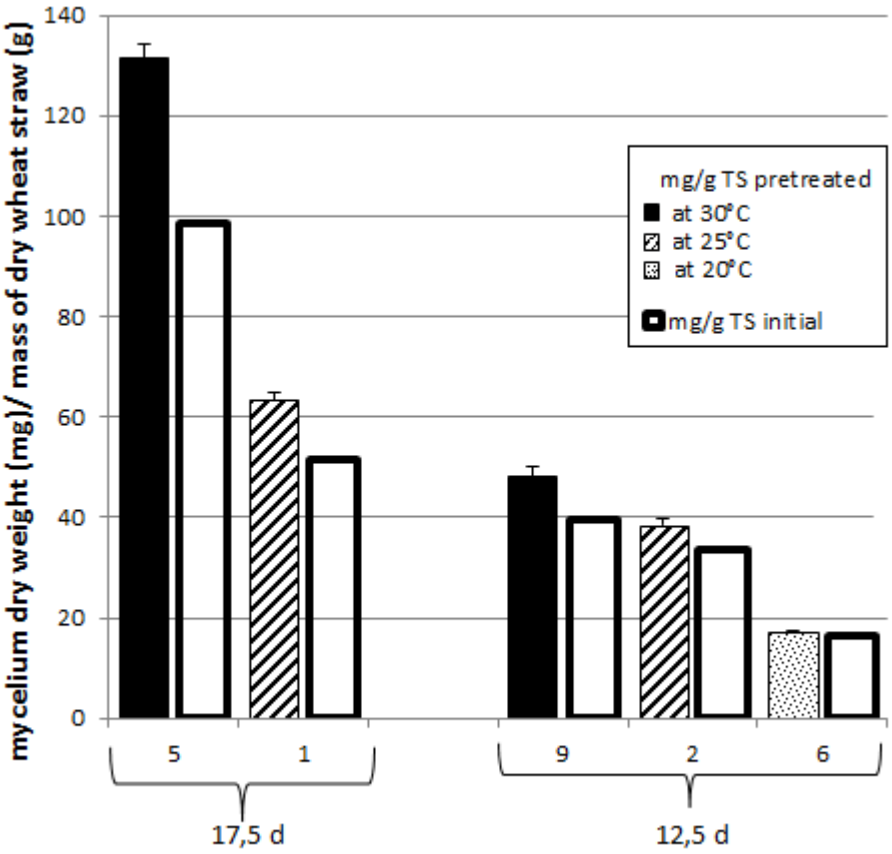


Figure 4-4. qPCR measurements of fungal biomass on straws pretreated with metals addition.

Samples with a 17.5-days culture duration have higher fungal biomass than those cultivated for 12.5 d (whatever the unit used: mg/g TS pretreated or mg/g TS initial, before pretreatment). It can especially be observed comparing samples 5 and 9 that were obtained in the same conditions (30°C, WW/TS = 3.7) except for pretreatment duration. qPCR quantifies DNA from alive cells but also from a part of dead ones (if their DNA is not lysed) (Cangelosi and Meschke, 2014). However, in this study it does not seem to be a limitation since methane production from fungal cells depends on their constituents (protein, carbohydrates…) and not of their viability. qPCR appears particularly adapted to estimate fungal biomass in early stage of decay, i. e. firsts weeks (Eikenes et *al.*, 2005), especially when the extraction step is efficient as it was the case for the current study (Zhou et *al.*, 2015a).

To fully investigate influence of mycelium amount on methane production, an estimation of fungal biomass in the whole experimental domain would be helpful. Fungal biomass is correlated to dry matter losses (DML) whatever the WRF strains (Shi et *al.*, 2014a). DML are correlated to qPCR results (R^2 = 0.8018, n=5, Figure 4-5A). Response surfaces (RS) were built for DML (Annex 4 and Annex 5). Those RS showed that DML (and probably fungal biomass) were almost independent of metals addition factor and increased with culture duration with a maximum between 18 and 19 days. DML were maximized near 28-29°C and

with WW/TS close to 3.3 (Table 4-6). Thus, fungal biomass would increase with rising temperatures (Figure 4-4 and Annex 4). DML were minimized for low durations, moisture content and temperature.

Methane production at day 6 (NmL/g VS $_{pretreated}$) was correlated to DML (R^2 = 0.6162, n=18, Figure 4-5B). Furthermore, methane production at day 57 (NmL/g VS $_{pretreated}$) was slightly correlated to DML (R^2 = 0.5065, n=18, Figure 4-5B), as well as BMP (NmL/g VS $_{pretreated}$) (R^2 = 0.4567; R = 0.6904 > 0.4821, threshold of significance for 18 points (DOF=15) with α = 0.05, Figure 4-5B). Since DML are probably representative of fungal biomass, correlations in Figure 4-5B would be linked to the fast degradation of fungal mycelium which principally occurs in the first days of BMP tests.

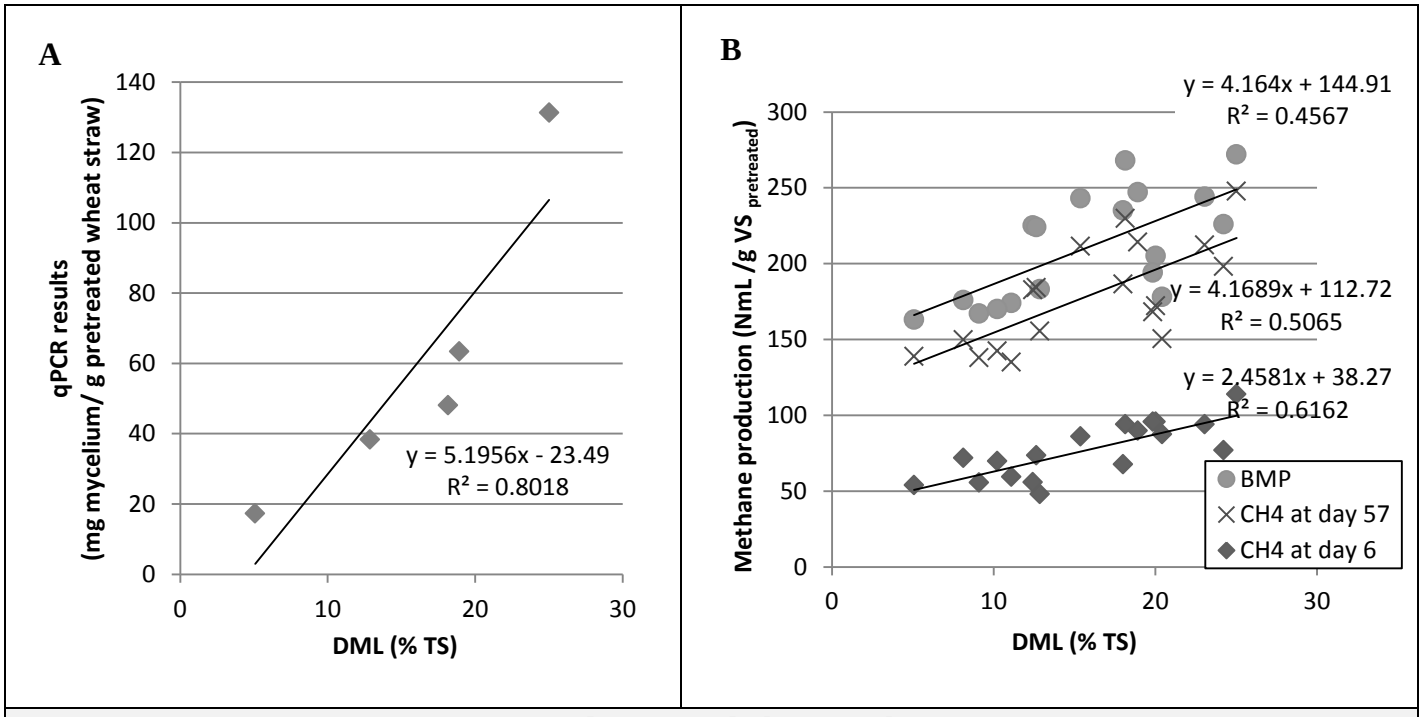


Figure 4-5. Correlations with dry mass losses (DML).
A. qPCR versus DML. B. Methane production versus DML.

4.3.5 Contribution of cellulose degradation to methane production

Holocelluloses digestibility reflects the accessibility to anaerobic digestion substrate. After pretreatments for optimization step, S. Zhou measured glucose released after enzymatic hydrolysis. The enzymatic cocktail used (GC220 and β-glucosidase, see 4.2.3.5) contained few hemicellulases but xylose release is poorly efficient (Ravalason et *al.*, 2012). However, xylose is the major compound of wheat straw hemicelluloses. Thus, even if whole reducing sugars released after enzymatic hydrolysis were also measured by S. Zhou; this amount would not well represent holocelluloses digestibility that is why it was chosen to only study the link between cellulose digestibility and methane production. After the pretreatment, a correlation between cellulose and hemicelluloses losses was observed (R^2 = 0.8663, n=18, Chapter 7, Figure 7-6). Hemicelluloses are generally considered as more degradable than cellulose during anaerobic digestion, notably owing to their amorphous structure. However, cellulose degradation is sometimes more efficient than the one of hemicelluloses. Pakarinen et *al.* (2012) observed a preferential degradation of C6 (soluble

glucose and glucose from cellulose) on C5 degradation (from hemicelluloses) during a 30-days anaerobic digestion reaction. In Pakarinen et *al.* (2012) study's, the amount of soluble glucose (more accessible than cellulose) in the C6 fraction would not be sufficient to explain the greater degradation compared to C5 fraction. In fact, with sufficient nitrogen amount cellulose can be digested preferentially to hemicelluloses under mesophilic conditions (Ghosh et *al.*, 1985). During the BMP tests, nitrogen amount is assumed sufficient (as added in macro-elements solution) and cellulose may contribute more to BMP than hemicelluloses for pretreated straws.

BMP (NmL/g VS) and methane production after 57 days (NmL/g VS) were correlated to glucose yield after 72 hours of enzymatic hydrolysis (Figure 4-6A). Low glucose yields represented low matter losses (all polymers taken together) since DML were taken into account to calculate glucose yield. Low glucose yield also reflected a low accessibility of carbohydrates (caused by lignin, cellulose crystallinity…), that is why glucose yield and lignin amount were correlated (R^2 = 0.9461, n=18, Annex 6).

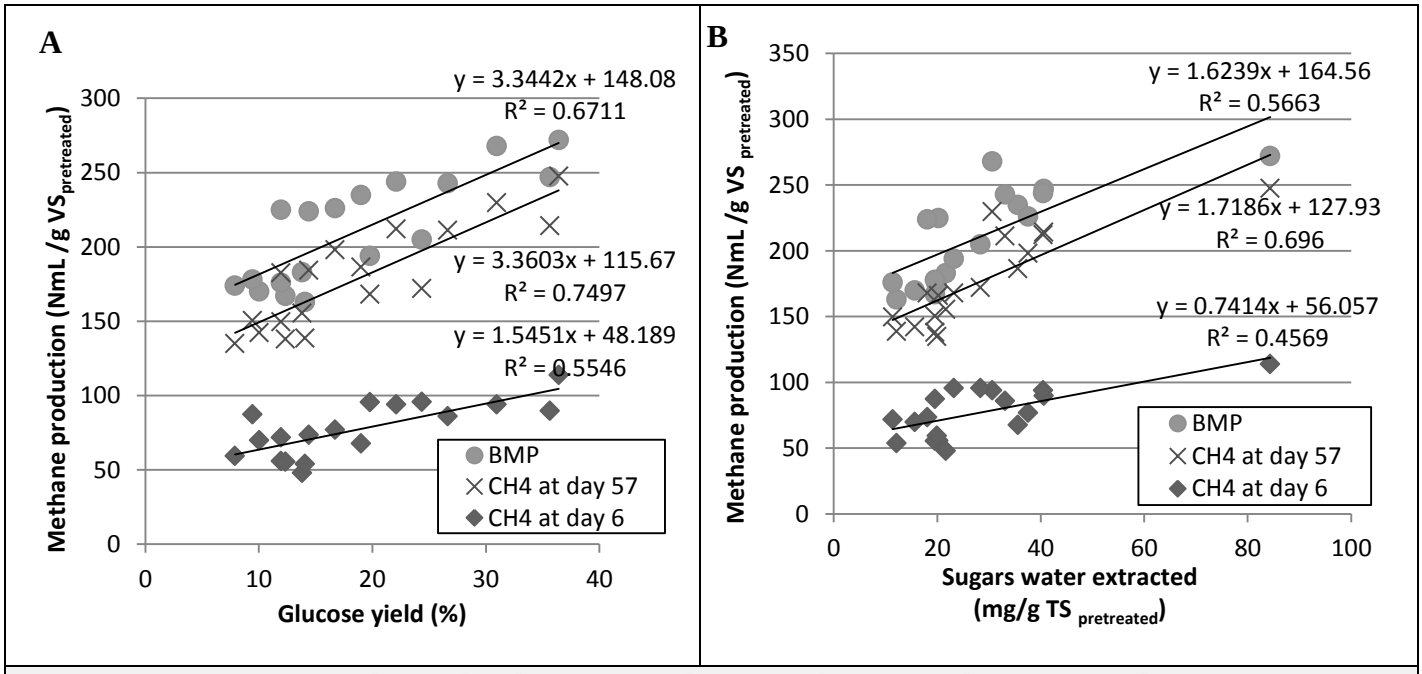


Figure 4-6. Relationship between methane production and sugars in substrate.
A. Methane production versus glucose yield from enzymatic hydrolysis. B. Methane production versus water soluble sugars.

Methane production at day 6 was slightly correlated to glucose yield (R^2 = 0.5546, n=18, Figure 4-6A). Cellulose and hemicelluloses are less accessible than proteins and some other carbohydrates (soluble sugars, extracellular polymeric substances…) (Jimenez et *al.*, 2015) that constitute fungal biomass. Thus, cellulose degradation (assessed with glucose yield) influences more methane production after 57 days. In contrast, fungal biomass (supposed correlated to DML) can be degraded from the beginning of the anaerobic digestion and is more linked to methane production after 6 days. In the first 6 days, cellulose would be poorly attacked compared to more accessible compounds since glucose yield was poorly related to methane production at day 6 (Figure 4-6A). The slight decrease of the correlation between glucose yield and BMP comparatively to CH_4 at day 57, is probably due to the fact that BMP is the resultant of all components

degradation (proteins, holocelluloses…). Moreover, hemicelluloses degradation was not assessed. During the long reaction duration necessary to obtain the BMP values (≈ 6 months), more compounds would be released than after the 72 h enzymatic hydrolysis (better accessibility, enzymes efficiency…). Furthemore, recalcitrant compounds can be transformed into methane for long-reaction duration, as already discussed.

It can also be noticed that water soluble sugars are moderately correlated to glucose yield (R^2 = 0.5928, n=18, Annex 6). Thus, water extracted sugars are correlated to methane production at 57 days (R^2 =0.6505, Figure 4-6B) but slightly to CH_4 at day 6 (R^2 = 0.4569, Figure 4-6B) and to BMP (R^2 = 0.5663, Figure 4-6B). Small water soluble sugars obtained in this study would slightly influence BMP (low slope and R^2). According to the model of Monlau (2012b) (Figure 4-1), maximum methane amount (NmL/g TS pretreated) due to soluble sugars would be 6.5 mL (for sample 5) that is not higher than the standard-deviation obtained in BMP measurements (Table 4-3). However, it must be noticed that in their study soluble sugars where extracted under mild acid conditions. Thus, water soluble sugars may not be enough representative of mild acid soluble sugars. Otherwise, their small amount would explain why their influence was slightly observed on methane production at day 6 although those compounds would not require extensive hydrolysis.

The moderate correlation between soluble sugars and glucose yield probably shows that solubilization of holocelluloses contribute to soluble sugars. Soluble sugars were also positively correlated to qPCR results (R^2 correlation=0.9971, n=5, Annex 6) and DML (R^2 correlation=0.5403, n=18, Annex 6). Those correlations would show that soluble sugars origin can also be the release of fungal extracellular polysaccharides (Gutierrez et *al.*, 1995). Thus, water extracted sugars would be directly linked to fungal pretreatment. Soluble sugars increase is often reported for fungal culture durations varying from 1 to 2 weeks (Agosin et *al.*, 1985; Shrestha et *al.*, 2008a) whereas a decrease is observed for longer times (from 2 to 4 weeks) (Agosin et *al.*, 1985; De Souza et *al.*, 2006) probably due to fungal consumption of sugars released from lignocellulosic biomass (Salvachúa et *al.*, 2013).

Study of glucose yield after enzymatic hydrolysis appeared relevant to study BMP and methane production at 57 days and RS for glucose yield were built (Annex 7 and Annex 5). Glucose yield was higher with metals and was mostly influenced by moisture content (high moisture content led to high glucose yield) (Table 4-6). Duration is slightly influent. Increased temperature (> ≈ 25°C) in combination with a sufficient moisture content (> ≈ 3.5°C) enhanced glucose yield.

Table 4-6. Extrema for RS of the current study: DML, lignin losses, glucose yield, methane production at day 6 and 57 and BMP.

Metals	Time (d)	WW/TS	Temp (°C)	Maximum calculated values	Minimum calculated values
Yes	18.68	3.21	28.59	DML = 28% TS	
No	18.36	3.38	28.86	DML = 28% TS	
Yes	18.18	3.81	28.62	Lignin losses = 34%TSi	
No	17.82	3.71	27.5	Lignin losses = 28%TSi	
Yes	16.49	4.29	28.83	Glucose yield = 42%	
No	16.16	4.42	28.34	Glucose yield = 30%	
Yes	15.85	4.15	29.67	CH_4 day 6 = 86 NmL/g TSi	
No	16.29	4.06	29.79	CH_4 day 6 = 72 NmL/g TSi	
Yes	11.67	4.02	28.22	CH_4 day 57 = 180 NmL/g TSi	
Yes	19.84	3.46	26.36	CH_4 day 57 = 182 NmL/g TSi	
No	10.32	3.76	25.78	CH_4 day 57 = 152 NmL/g TSi	
No	19.86	3.15	23.73	CH_4 day 57 = 144 NmL/g TSi	
Yes	11.37	4.00	27.84	BMP = 206 NmL/g TSi	
Yes	19.99	3.22	25.04	BMP = 213 NmL/g TSi	
No	10.31	3.74	25.85	BMP = 185 NmL/g TSi	
No	19.54	2.98	22.91	BMP = 169 NmL/g TSi	
Yes	13.61	2.97	19.41		DML = 4% TS
No	13.80	2.83	19.54		DML = 2% TS
Yes	12.23	2.78	20.52		Lignin losses = 6%TSi
No	12.84	2.93	19.82		Lignin losses = 2%TSi
Yes	12.78	2.09	26.37		Glucose yield = 14%
No	13.99	2.17	28.30		Glucose yield = 6%
Yes	17.12	3.47	19.62		CH_4 day 6 = 47 NmL/g TSi
No	17.36	3.91	20.39		CH_4 day 6 = 45 NmL/g TSi
Yes	13.92	3.12	19.19		CH_4 day 57 = 121 NmL/g TSi
No	15.22	4.07	19.98		CH_4 day 57 = 118 NmL/g TSi
Yes	13.48	3.18	19.31		BMP = 142 NmL/g TSi
No	14.92	3.81	19.42		BMP = 141 NmL/g TSi

4.4 Response surfaces for methane production

To further investigate implied mechanisms in methane production from pretreated straws and to find optimum pretreatment conditions to maximize methane production; RS for methane productions at day 6, day 57 and BMP were built (models coefficients in Annex 5). All RS of the current studies were significant (Table 4-7) at least at a 90% confidence level (p-value < 0.1).

Table 4-7. Sums of squares for regression and residues, statistic F and p-value for built response surfaces.
***<0.001; **<0.05;*<0.1.

	DOF	DML (%TS)	CH_4 d 6 (NmL/g TS i)	CH_4 d 57 (NmL/g TS i)	BMP (NmL/g TS i)	Lignin losses (%)	Glucose yield (%)
Regression	13	578.98	2386.39	7146.22	7849.09	1277.85	1311.54
Global residuals	6	8.64	41.38	978.14	359.76	15.88	27.49
Residuals	4	7.32	27.61	388.48	738.60	14.16	24.11
Error	2	1.32	13.77	589.66	1419.97	1.72	3.37
F statistic	-	30.92	26.62	3.37	10.07	37.15	22.02
p-value	-	2.07E-04***	3.19E-04***	7.22E-02*	4.84E-03**	1.21E-04***	5.49E-04***

In the current study, response surfaces were calculated for CH_4 yields expressed per g of initial TS (before pretreatment) since they are the most relevant to optimize for industrial applications as they take into account mass losses. RS for methane productions without taking into account mass losses were also studied (NmL/g $VS_{pretreated}$) but did not allow to better explain mechanisms, probably because methane production expressed in different units were correlated (Figure 4-7).

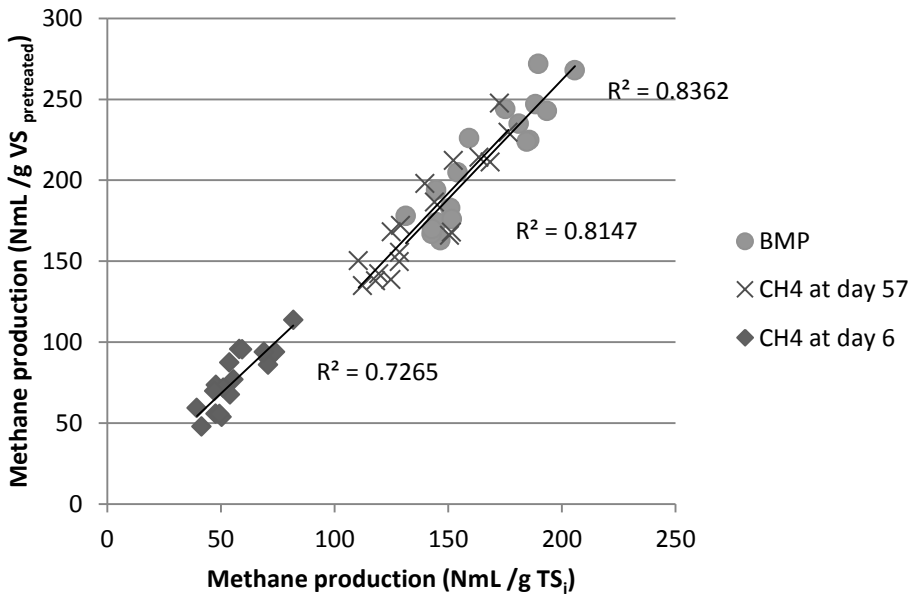


Figure 4-7. Correlation between methane productions expressed per gram of VS pretreated or per gram of TS initial.

4.4.1 Methane production after 6 days

Apart from fungal biomass, lignin content and sugars yield, other unidentified structural features were likely implied in methane production at day 6 (since R^2 were not extremely high < 0.65 for the three correlation, see 4.3). RS for this variable (Figure 4-8) showed a small positive influence of metal addition on CH_4 production (as for lignin content and sugars yield). It also gave highest values for high temperature and water amount (in accordance with RS for lignin losses, DML and sugars yield). However, the maximum was obtained between day 15-16, close to optimized culture duration for glucose yield after enzymatic hydrolysis whereas maximum DML and lignin losses require 18-days culture duration (Table 4-6). Since culture duration is only slightly influent on glucose yield, other parameters would explain the maximum methane production after 6 days of BMP-tests for culture duration close to 16 days.

A positive influence of WW/TS, especially for short culture duration, was noticed. It can be related to a limitation of nutrients and enzymes circulation with small water amount. Without metal addition, WW/TS had a lower influence on methane production at day 6. A slight lower influence of moisture without metals was also visible on RS of lignin losses. With RS of DML, lignin losses and sugars yield, temperature had generally a higher influence than moisture content. It is also the case for RS of methane production at day 6 (increasing with enhanced temperature). As for lignin losses and DML, low methane productions at day 6 were obtained for small culture duration and low WW/TS.

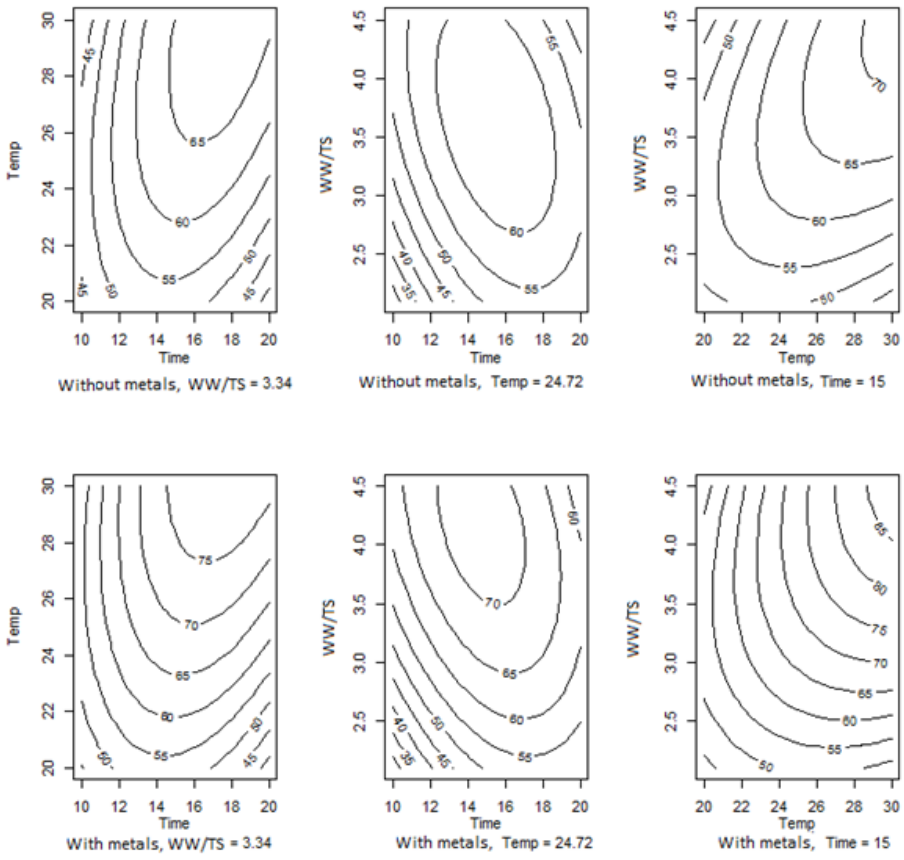


Figure 4-8. RS for methane production at day 6 (NmL/g TSi).
Contour plots correspond to methane production at day 6 depending on pretreatment parameters: Temp: Temperature in °C; WW/TS: initial moisture content in g/g; and Time: duration in days.

4.4.2 Methane production after 57 days

RS for methane yield after 57 days of anaerobic digestion (NmL/g TSi) showed a positive influence of metal addition (Figure 4-9) as for delignification and glucose yield after enzymatic hydrolysis (shown influent on this methane production).

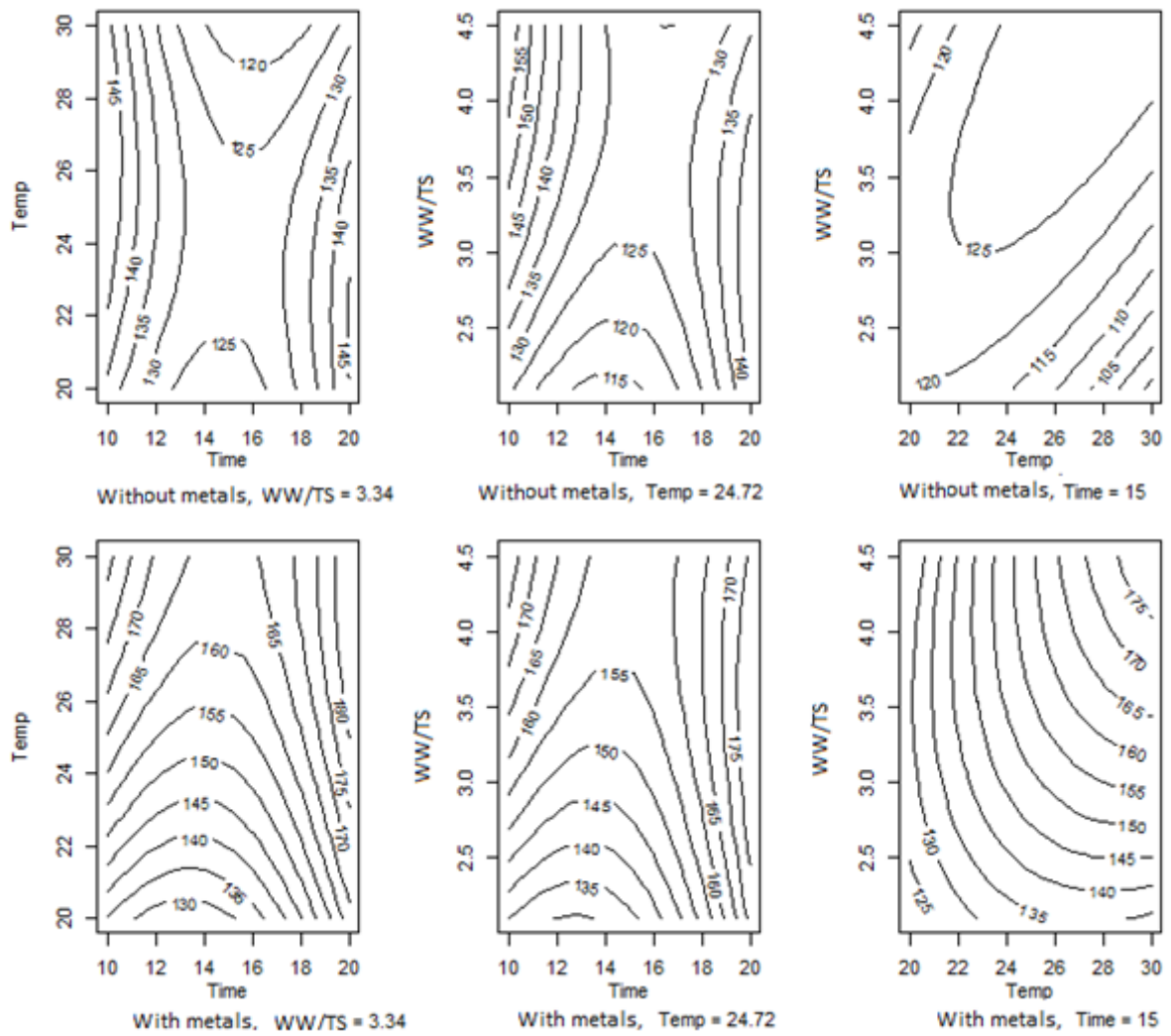


Figure 4-9. RS for methane production at day 57 (NmL/g TSi).
Contour plots correspond to methane production at day 57 depending on pretreatment parameters: Temp: Temperature in °C; WW/TS: initial moisture content in g/g; and Time: duration in days.

4.4.2.1 Samples pretreated with metals addition

After metal addition, culture duration was the second most influent parameter of RS for methane yield at 57 days (/g initial TS). In Figure 4-9, it can be observed that greatest variations of methane production at 57 days correspond to culture duration changes. The optimal methane production at 57 days, i. e. the maximum obtainable methane production with the fungal pretreatment, is not reached in the experimental domain. However, highest methane productions were observed for short (≈11 d) and long (≈19 d) durations (Table 4-6).

The maximum methane production for long culture duration for samples with metals addition was due to high lignin losses and high glucose yield (enzymatic hydrolysis) for those durations. It was also highlighted that fungal biomass amount (estimated with DML) was probably maximal for long culture duration and contributed positively to methane production. For long culture duration, increased temperature had slightly more positive influence than increased moisture content on methane production at 57 day (Figure 4-9).

High values of methane production were also obtained for small durations of pretreatment. For small pretreatment durations (with metals), it seems that fungal biomass also had a positive influence since highest methane production were obtained with highest fungal biomass (Sample 6 < Sample 2 < Sample 9) (Figure 4-4 and Table 4-3). DML and lignin losses were maximized with high temperature for short duration. Moisture content was less influent (especially on lignin losses). Highest values of DML corresponded to moisture content close to 3.5 (Annex 4). For short durations, good glucose yield after enzymatic hydrolysis can be obtained with high temperature and moisture content (Annex 7). Thus, high temperature and moisture content also maximized methane production at 57 days for short duration (Table 4-6).

Culture conditions leading to minimum calculated values for methane production at 57 day were close to those minimizing lignin losses and DML: small temperature and duration between 12-14 d.

4.4.2.2 Samples pretreated without metals addition

Even if added metals amount is small, it will constitute an additional cost at industrial scale. This cost is not crippling if methane increase is consequent. Notwithstanding, substrate modifications and methane production without metals addition must also be studied. Without metals, calculated lignin losses are in average 5% lower than with metals addition (Annex 3). Without metals, response surfaces for methane production at day 57 were similar to those with metals, except that maximum value is only found for small duration (Figure 4-9). Assumptions on the pretreatment action outside of the experimental domain are not possible without complementary experiments (allowing an increased experimental domain). However, RS for lignin losses inside the experimental domain show that approximately two days more without metals addition are required to reach same delignification levels as those obtained with metals addition (Annex 3). It shows the positive influence of metals on fungal delignification-rate. The maximum delignification level seems to be reached inside the experimental domain (Annex 3). The absence of maximum calculated BMP value for long culture duration without metals can be explained by similar DML for long-culture duration with or without metals but lower lignin losses without metals. Thus, sugars losses were probably increased compared to sample with metals for long-culture duration.

Fungal biomass amount (represented by DML) was more influent on methane production rate than on methane yield (Figure 4-5B). DML are taken into account to evaluate the global efficiency of the process (pretreatment and anaerobic digestion) by expressing methane productions per gram of initial TS. Thus, DML must be moderate. However, lignin losses were shown very important to increase methane production. High lignin losses correspond to high DML. In fact, the selectivity for lignin losses must be considered and DML without information on lignin losses is not enough informative. Notwithstanding, BMP (NmL/g TSi) for two pretreated straws having similar lignin losses while be maximized for the sample having the lower DML. Importance of lignin selective attack can also be observed for samples with metals for long culture duration: lignin losses were identical above ≈ 25°C whereas DML continued to increase with rising temperatures (Annex 3 and Annex 4). It means that above ≈ 25°C for long-culture duration sugars losses increased. It explains why smaller culture temperature maximizes methane production for long culture duration compared to temperature required for short culture duration (Table 4-6).

4.4.3 BMP

Methane production at day 57 and BMP had similar behavior regarding lignin content, DML and glucose yield after enzymatic hydrolysis (see 4.3). Thus, RS for BMP (Figure 4-10) had a similar behavior than RS for methane production at 57 days: significant positive influence of metals addition and presence of two maxima with short and long culture durations (with metals), respectively. The maximum for long culture duration with metals addition is also higher than the one for short duration (Table 4-6). Implied mechanisms discussed for methane production at day 57 would be also valuable for BMP. Temperatures for maximum BMP with short-culture duration were highest than for long-culture duration (as with methane production at day 57). Without metals, maximum calculated BMP value corresponded to short culture duration (Table 4-6). Temperature for this maximum was lower than the one for maximization of BMP with metals for short culture duration. This was likely linked to the little influence of temperature on glucose yield after enzymatic hydrolysis (influencing BMP) without metals and for short culture duration (Annex 7).

For samples with metals and for long culture duration, temperature and moisture content have little influence on the BMP (Figure 4-10). However, high values for temperature and WW/TS allow an increase BMP for the short culture duration (30-40 NmL/g TS i more). To increase BMP, elevation of temperature during the pretreatment appears slightly more efficient than moisture content increase for short duration. It is also the case for lignin losses (Annex 3).

Maximum calculated BMP value (239 NmL/g TS i, Table 4-6) is not very different from the measured BMP of NIC (≈227 NmL/g TS i) but is better than measured BMP for untreated wheat straw (183± 5 NmL/g TS). Parameters used for pretreatment of sample 9 are the closest ones from the maximum calculated BMP for short duration (at 10.63 d) (Table 4-6). Measured BMP of this sample is among the best ones (Table 4-3). According to the response surfaces for samples with metals addition, the minimum calculated BMP (144 NmL/g TS i) is obtained with culture parameters very close to those of samples 6 that had effectively one of

the worst measured BMP. Minimum calculated value for lignin losses with metals addition (Table 4-6) was obtained for pretreatment conditions very close to those leading to the minimum calculated BMP and to one of the minimum measured BMP with metals (sample 6).

In the current study, it was highlighted that pretreatment conditions were not optimal and did not allow a sufficient BMP improvement. Thus, effects on biodegradability were limited. The highest calculated values for BMP was 213 NmL/g TSi (Table 4-6), it is not different from BMP values obtained for NIC (216 NmL/g TS) but better than BMP of UWS (183 NmL/g TS). Maximum values were generally obtained with WW/TS between 3 and 4, that was already considered as optimal for wheat straw pretreated with WRF (Zadražil and Brunnert, 1981).

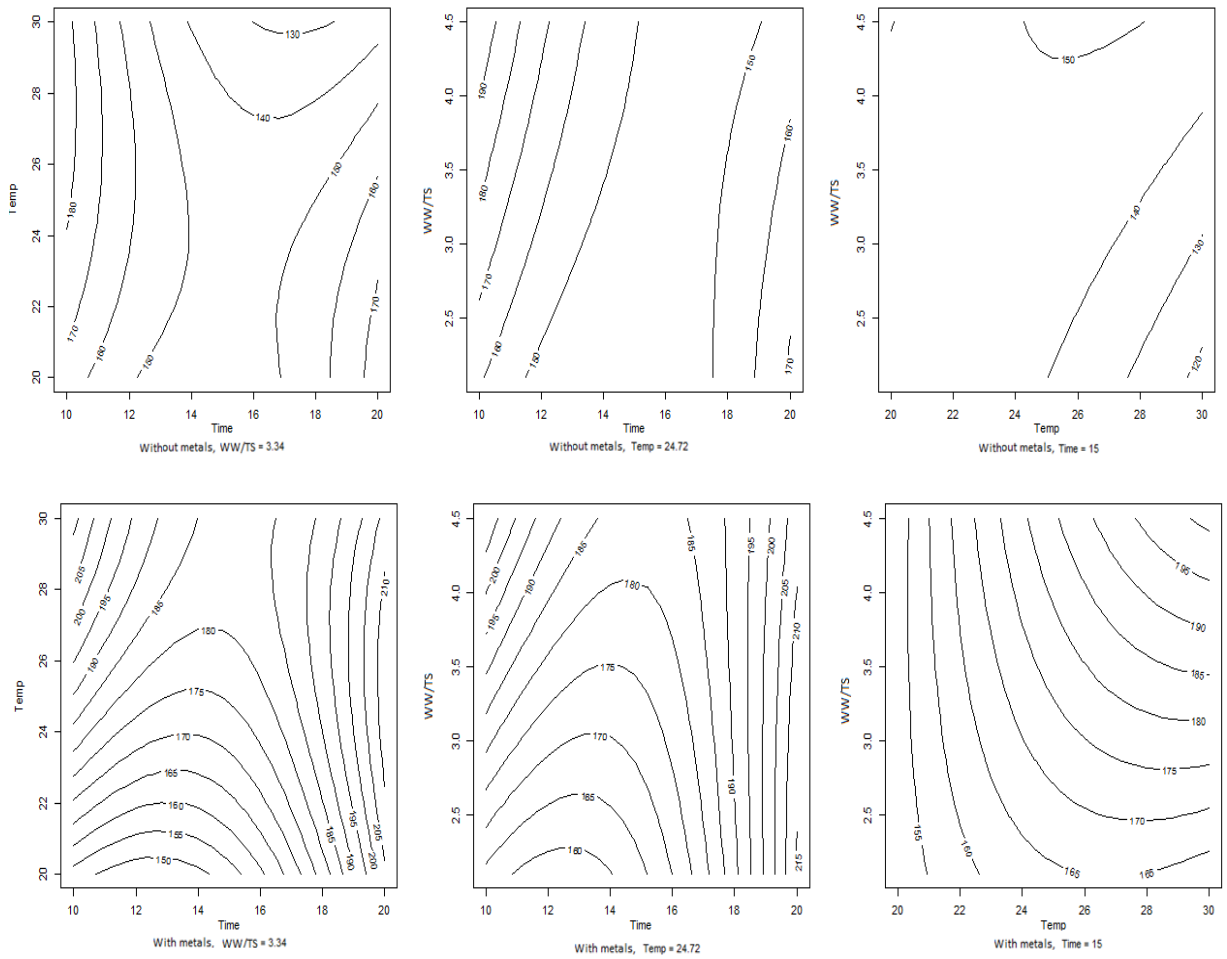


Figure 4-10. RS for BMP (NmL/g TSi).
Contour plots correspond to BMP depending on pretreatment parameters:
Temp: Temperature in °C; WW/TS: initial moisture content in g/g; and Time: duration in days.

4.5 Conclusion

Metals addition enhances lignin decomposition by fungi. Increased lignin decomposition improves enzymatic hydrolysis of cellulose and methane production from the substrate. Fungal biomass, easily and rapidly degradable, would increase methane production rate and influence positively but slightly the BMP value of pretreated substrate. The second most important pretreatment parameter to increase methane production was culture duration. With metals addition two maxima for BMP (NmL/g TSi) were observed for short and long culture duration, respectively. It would allow a certain flexibility of the pretreatment duration that can be advantageous for industrial application.The absence of maximum for long-culture duration without metals is probably due to a less selective delignification. Best pretreatment duration in those conditions is still unknown. Pretreatment temperature and initial moisture content of the substrate must be sufficient even if those parameters have a lower influence on subsequent methane production. In the tested conditions, it seem possible to work with fixed temperature and moisture content. According RS for BMP (Figure 4-10), it is proposed to use 25-26°C and WW/TS = 4.

Chapter 5.

Pyrolysis-GC-MS study of White-Rot Fungi pretreated wheat straws for anaerobic digestion

Objectives

- To determine the interest of py-GC-MS technique to investigate fungal pretreated wheat straws for anaerobic digestion and, notably the possibility to use py-GC-MS to access pretreatment efficiency
- To understand the link between py-GC-MS data and data from more usual measurement techniques

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Highlights

- High protein (containing phenylalanine) and carbohydrate (glucose polymers) amounts in mycelium of *P. brumalis* BRFM 985
- Suitability of py-GC-MS to study S/G ratio
- Possibility to estimate fungal biomass amount on WRF pretreated straws with py-GC-MS
- Possible usefulness of polysaccharides/lignin ratio to select efficient strain for fungal pretreatment
- Possibility to access strain efficiency thanks to polysaccharides/lignin ratio determined with py-GC-MS

5.1 Introduction

Galletti & Bocchini (1995) defined pyrolysis as the thermal fission (break) of a sample in the absence of oxygen into molecules of lower mass, low enough to be suitable for GC and/or MS and large enough to provide analytical information on the original sample. Fragments are separated and identified thanks to GC-MS. Their nature and relative distribution constitute a fingerprint characteristic of a particular sample. Py-GC-MS is considered as a sensitive, rapid and reproducible technique with almost no waste generation (Galletti and Bocchini, 1995). Moreover, analysis duration is short compared to other existing methods (Alves et *al.*, 2006).

Some compounds formed by Py-GC-MS are known to originate specifically from lignin, polysaccharides, protein, etc. Thus, Py-GC-MS of lignocellulose was successfully **applied to study** relative amounts of lignin and carbohydrates, to classify lignin based on their S/G ratio, to follow chemical changes in cell walls during plant maturation or after chemical or biological delignification. Finally, it was used to evaluate the origin and composition of food and feed, forest litter, compost and materials from paper industry (pulps, papers, effluents) (Galletti and Bocchini, 1995). Pyrolysis is generally a semi-quantitative method but some studies established a correlation between Klason lignin and lignin estimated with pyrolysis for lignocellulosic biomass (Alves et *al.*, 2006; Fahmi et *al.*, 2007; Ross and Mazza, 2011).

Few studies were carried out on WRF pretreated wheat straw using py-GC-MS (Camarero et *al.*, 1994; Galletti and Bocchini, 1995). Application potential of this technique seems not yet fully exploited. In particular, **estimation of fungal biomass** amount with py-GC-MS would be interesting in order to control the fungal growth during pretreatment. A recurrent difficulty with fungal biomass measurement methods is the frequent variation of conversion factors with fungal species but also within a single species (due to environmental growth conditions) (Zhou et *al.*, 2015a).

Methane production from lignocellulosic biomass can be predicted using biomass contents of: lignin and crystalline cellulose (negative impact), amorphous holocelluloses and protein (positive impact) (Figure 4-1). With the same straw, protein content of fungal pretreated straws is mainly a function of fungal biomass amount that was shown to influence positively anaerobic digestion of fungal pretreated substrate (Chapter 4). **Crystallinity** of cellulose can influence cellulose behavior under pyrolysis (Zhang et *al.*, 2010; Zhu et *al.*, 2004), amorphous regions would produce less characteristic fragments. Consequently, the ratio **polysaccharides/lignin** (PS/LIG), with PS corresponding to holocelluloses, seems a good indicator for the valorization of a WRF pretreated lignocellulosic substrate and for the evaluation of the pretreatment effectiveness. Measurement of **S/G** (synapyl /guaiacyl) ratio can also be helpful to study anaerobic digestibility since S/G ratio influences lignin degradability (Monlau et *al.*, 2013).

The **aim** of this study was to investigate the suitability of py-GC-MS to assess the anaerobic biodegradability of wheat straw pretreated by several fungal strains (five WRF and one Brown-Rot Fungi

(BRF)). Firstly, mycelium of the WRF *P. brumalis* BRFM 985, fungal treated and untreated straws were analyzed with py-GC-MS and their pyrolysates were described. Secondly, relationships between pyrolysis data, other characterization technics and anaerobic degradability were studied, notably thanks to a Principal Component Analysis (PCA).

5.2 Material and methods specific to py-GC-MS study of pretreated straws

5.2.1 Fungal pretreated samples

As previously described (see 3.2.1.2), pretreatment of wheat straw was realized in glass columns. Samples for Py-GC-MS correspond to those used for selection step (Chapter 1) with three weeks of pretreatment. Two samples more were added, corresponding to pretreatment with strains BRFM 985 and BRFM 1554 with a culture duration of two weeks (suffix '-2w' added to the BRFM number for the identification of corresponding samples). These two samples were inoculated with 130 mg (dry weight) ground mycelium whereas others received 120 mg (dry weight). At the beginning of the cultivation, straw was moistened: wet straw weight corresponded to 4.5 fold initial TS weight for samples pretreated three weeks and 4.25 for samples pretreated two weeks. Samples (Table 5-1) were identified thanks to their BRFM number in Py-GC-MS results (Chapter 5). Some samples issued from the optimization of pretreatment with *Polyporus brumalis* BRFM 985 were also characterized with Py-GC-MS. Those samples (Table 5-1), corresponding to several culture conditions, were identified by a number preceded by the letter C. Some of them received a metals solution at the inoculation composed of $MnSO_4$; $CuSO_4$ and $FeSO_4$ at 18 mM each. Three temperatures of culture (20°C, 25°C, 30°C), two culture durations (12.5 or 17.5 d) and four initial Wet Weight (WW) were used.

Table 5-1. Pretreated wheat straws studied with Py-GC-MS.

Samples	Strains	Metals addition	Culture duration (d)	Temperature (°C)	Initial WW/TS
1554	*Trametes pavonia*	No	21	28	4.25
1554-2w			14		4.5
957	*Trametes ljubarskii*		21		4.25
236	*Gloeophyllum trabeum*				
1296	*Trametes cingulata*				
1048	*Leiotrametes sp.*				
985	*Polyporus brumalis*				
985-2w			14		4.5
C1		Yes	17.5	25	4.5
C2		Yes	12.5	25	2.1
C9		Yes	12.5	30	3.7
C13		No	17.5	25	4.5
C15		No	17.5	20	2.9
C17		No	12.5	30	3.7

5.2.2 Pyrolysis-GC-MS

Py-GC-MS were realized at the INRA of Grignon (ECOSYS UMR) with the help of MF Dignac.

5.2.2.1 Spectra obtaining

Prior to the pyrolysis-GC-MS analysis, samples were ground for 5 min at 25 Hz (Mix Mill 400, Retsch). Straws pretreated with several strains (selection step), with a culture duration of three weeks or two weeks ('-2w') and untreated straw (UWS) were analyzed with Py-GC-MS. Moreover, mycelium of BRFM 985 was also studied (cultivated in liquid medium, see 2.1.3). Triplicates were measured for straw samples. Only one mycelium sample was pyrolyzed as considered more homogeneous than straws.

Approximately 0.5–1 mg of sample was loaded in a quartz tube (2 mm x 25 mm) closed with quartz wool (B) and heated at 650°C in 0.15 s (30 s hold) with a pyrolysis unit (CDS Pyroprobe 5000 Series, A). At 650°C, all wheat straw polymers are attacked (Figure 1-19). Moreover, Wang et *al.* (2012a) recommended almost 600°C for cellulose pyrolysis. Pyrolysis products were transferred in splitless mode to a Hewlett Packard HP-6890 gas chromatograph (GC) equipped with a 60 m fused silica capillary column SolGelWax (SGE, 0.32 mm i.d., film thickness 0.5µm), with helium as carrier gas (1 mL/min) .

The temperature program of the GC oven was set with an increase from 30 to 280°C at 2°C/min, with the temperature maintained at 280 °C for 15 min. The GC was coupled to a Hewlett Packard HP-5973 mass spectrometer (electron energy 70 Ev.).

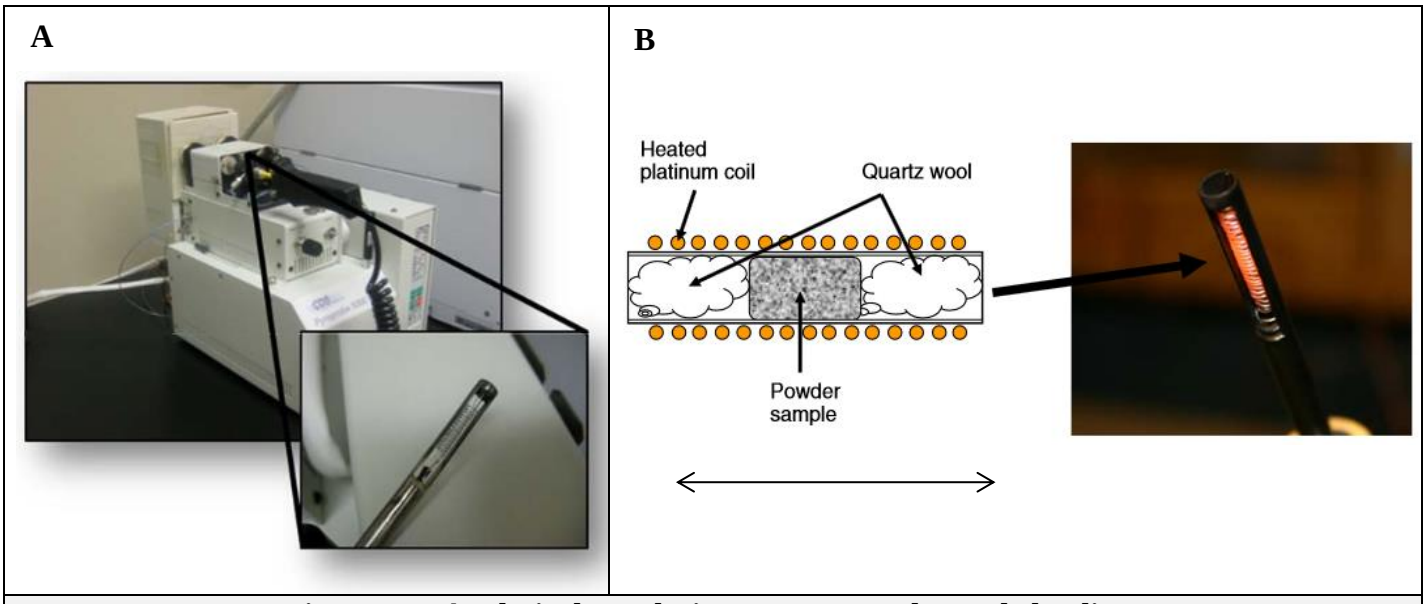


Figure 5-1. Analytical pyrolysis apparatus and sample loading.
A. CDS pyroprobe (Ware, 2013). B. Sample loading into quartz tube before insertion in the platinum coil (Carlson et *al.*, 2009).

5.2.2.2 Spectrum analysis and interpretation

Compounds in pyrolysates were identified on the basis of their mass spectra, GC retention times, and comparison with Wiley library mass spectra. Peaks were integrated using the HP MS Chemstation (Version C.01.05) on the total ion current trace. All pyrograms are displayed with phenol, 2,6-dimethoxy- (coded LIG6-S) having the same peak height. Compounds with a quality almost equals to 60% were taken into account (estimated by the software comparing library and measured mass spectrum). Other compounds were added and research manually (triplicates comparison, notably) to improve analysis precision. Relative areas per thousand of total area of spectra were calculated per sample for each molecule. An average of relative areas per compound was calculated for triplicates. Average relative areas per thousand were used to sum certain group of compounds or to obtain ratio with chosen compounds (results in Table 5-2). For these calculations, few values were excluded when too different from the two other replicate values (as mentioned in Table 5-2).

Samples issued from the optimization step (samples identified with letter "C") were extra-samples used to improve correlations robustness. For those samples, only few compounds were researched on pyrolysates and their peaks were integrated by the software (results in Table 5-3) without manual verification (based on triplicate comparison for peaks integration and identification). To calculate polysaccharides/lignin (PS/LIG) ratio, all compounds detected automatically by the software with a quality superior to 60% (without manual verification), were taken into account.

5.2.3 Characterization of fungal pretreated straws for anaerobic digestion

5.2.3.1 Acid hydrolysis (Klason lignin, cellulose and hemicelluloses) with NREL method

As previously described (in 3.2.3.2), the lignin, cellulose and hemicelluloses contents were analyzed by S. Zhou following adapted NREL two-stage acid hydrolysis method.

5.2.3.2 Qpcr

As already presented (see 4.2.3.6) fungal biomass was quantified with qPCR for few samples from the optimization step (C1, C2, C9). In their study, Zhou et *al.* (2015a) had estimated fungal biomass amount for samples pretreated two weeks but standard curves were different than for samples from the optimization step: standard curves were established for each strain using serial dilutions of genomic DNA ranging from 1 ng/µL down to 1.10^{-4} ng/µL. Zhou used the same methodology to measure samples from the selection steps (pretreated three weeks).

5.2.3.3 BMP-tests

Samples pretreated three weeks for selection step were measured in the same BMP-test whereas samples pretreated two weeks (985-2w and 1554-2w) were measured together in another BMP-test. Samples from the optimization step were measured together in a third BMP-test.

5.2.4 Statistical analysis

5.2.4.1 Analysis of Variances (ANOVA)

Analysis of variance (ANOVA, $\alpha = 0.05$) was carried out using R software (version 3.2.1) with "lattice" and "lawstat" libraries. Multiple means comparisons were performed with Tukey's HSD (Honest Significant Difference) test at the same significance threshold of 0.05.

5.2.4.2 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was built with SIMCA software from UMETRICS. Number of components is automatically fixed (based notably on Kaiser criterion). Quality representation for individuals (samples) is based on $Dmod_x$ that represents the samples distance to the model in the X-space (space defined by variables on retained component). $Dmod_x$ is expressed in normalized units when divided per the residual standard deviation of a sample to the model. The software plots it in a chart that allows detection of outliers ($Dmod_x$ > F crit) since statistical distribution is known (F-distribution) (Wikstrom et *al.*, 1998).

5.3 Origin of the main compounds found in pyrolysates

Compounds observed in the pyrolysates were classified according to their origin into several groups: polysaccharides derived products (PS), N-containing compounds (N), lignin-derived products (LIG). Another group was used for compounds with unspecific origin (UN) which can be formed from several sources during pyrolysis. The code attributed to main chemical compounds identified in pyrolysates is composed of an abbreviation of the origin (PS, N, LIG or UN) followed by a number (Annex 8 and Annex 9). Finally, compounds with unknown origin were just numbered: e. g. 64 for ethylbenzene and 66 for xylene.

5.3.1 Fungus characterization

Pyrogram of the mycelium of *Polyporus brumalis* BRFM 985 is presented in Figure 5-2. It is dominated by molecules with a polysaccharide origin (PS). Several N-containing compounds are also identified (N), they can have a protein origin or a chitin origin (Buurman et *al.*, 2007; Dignac et *al.*, 2005).

Chitin is specific to fungi and arthropod invertebrates. Chitin, a polymer of N-acetylglucosamine, has a structure similar to glucose but with a N-acetyl group replacing the hydroxy methyl group. Chitin amount in wood decaying fungi is variable, e. g. 2.6% or 26.6% of the dry cell-wall (Chen and Johnson, 1983).

Fungal dry weight is constituted in majority by carbohydrates and proteins and in minor amount by minerals, fat and pigments (Hadar and Cohen-arazi, 1986; Mohaček-Grošev et *al.*, 2001). Thus, pyrogram of *P. brumalis* BRFM 985 is in agreement with fungal composition in literature.

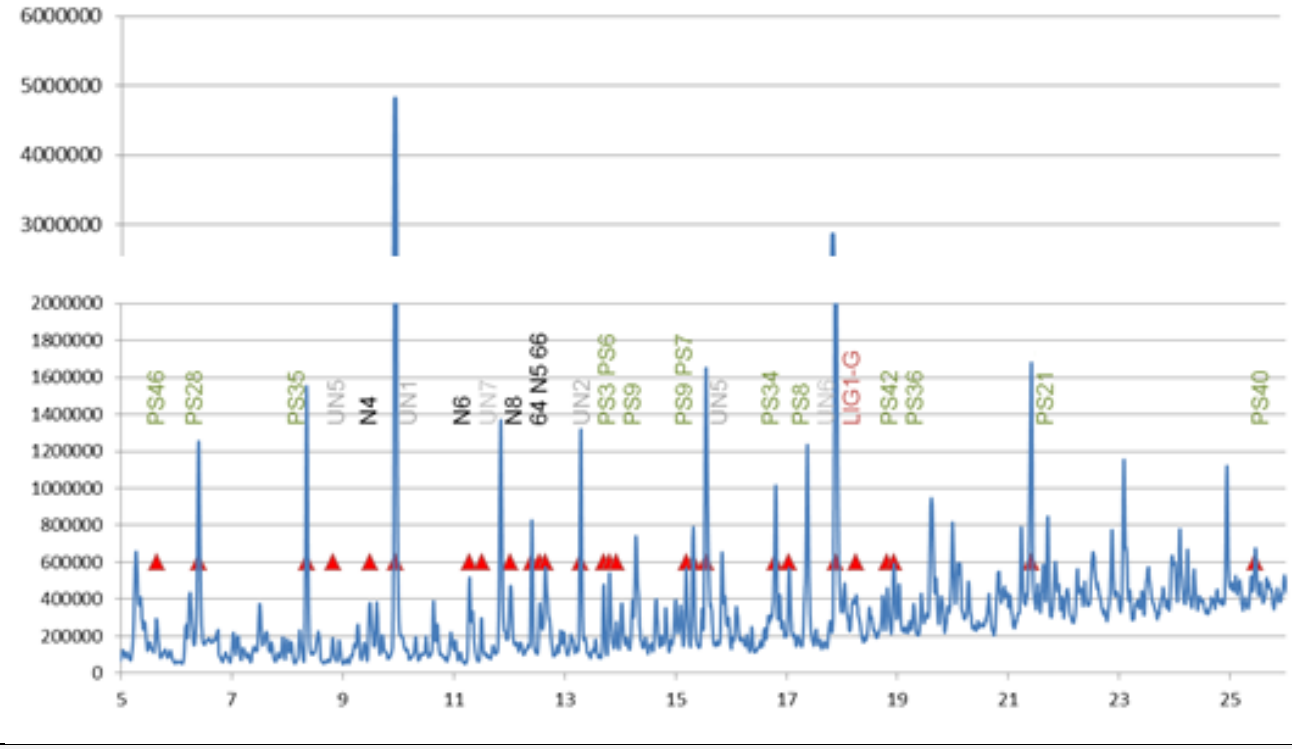


Figure 5-2. Pyrogram of mycelium of *Polyporus brumalis* BRFM 985.
Compounds codes are reported in Annex 9 (for compounds 64 and 66) and Annex 8 (all other compounds).

5.3.1.1 N-containing (N) and unspecific compounds (UN)

The most probable origin for N-containing compounds found in the mycelium pyrogram (Figure 5-2) was investigated. Pyridine (N4, N5) can derived from chitin pyrolysis or from proteins containing alanine (Buurman et *al.*, 2007; Dignac et *al.*, 2005).

Indoles (N1, N2) probably have a tryptophan origin in the fungus pyrolysate. Pyrrole (N8) can have whether a protein origin (hydroxyproline, proline, glycine and glutamic acid) or a pigments origin (Dignac et *al.*, 2005). Given the fact that pigments are minor compounds in fungi, the protein origin is the most likely.

Toluene (UN1), 4-methyl phenol (UN6) and phenol (UN5), are considered as unspecific (UN) because besides their protein origin, they can be minor peaks in lignin or tannins pyrograms (Dignac et *al.*, 2005). A lignin or tannin origin is unlikely for this fungus sample. Toluene (UN1) can also have a PS-origin (Ross and Mazza, 2011) and was detected in xylan fractions (Pouwels et *al.*, 1987). Phenol (UN5) and 4-methyl phenol (UN6) were minor compounds of cellulose or xylan pyrolysates (Nowakowski and Jones, 2008), but their polysaccharide origin was not strongly established. They could also originate from the presence of lignin in xylan (strong hemicelluloses-lignin linkages, difficult isolation of pure xylan fraction…) (Chimphango et *al.*, 2012; Monlau et *al.*, 2013). In the mycelium pyrolysate, toluene (UN1), 4-methyl phenol (UN6) and phenol (UN5) would be mainly due to proteins.

The origin of styrene (UN2) is unspecific (Dignac et *al.*, 2005). Significant contribution is found after pyrolysis of BRFM 985 mycelium. Apart from proteins, other possible origins for styrene (UN2) are p-coumaric or ferulic acids, lignin or tannins (Dignac et *al.*, 2005; Ralph and Hatfield, 1991). Since these

molecules are not major fungal compounds, a protein origin (phenylalanine) is also favored for mycelium pyrolysate. Phenylalanine pyrolysis leads to a high toluene (UN1) peak, a medium styrene (UN2) peak and a small ethylbenzene (64) peak (Chiavari and Galletti, 1992), which corresponds to the distribution of these three peaks in the fungus pyrolysate (Figure 5-2). Thus, they very likely indicate the presence of high phenylalanine amount in mycelium. Then, a tyrosine origin can be suggested for 4-methyl phenol (UN6) and phenol (UN5). UN1, UN2, UN5 and UN6 were main peaks in the fungus pyrolysate. Therefore, phenylalanine and tyrosine would be major amino acids in *P. brumalis* BRFM 985 mycelium.

By way of comparison, they were found in high quantity along with threonine in the WRF *Lentinus tigrinus* (Lechner and Papinutti, 2006) whereas histidine, aspartic and glutamic acids were main amino acids in the WRF *Pleurotus ostreatus* mycelium (Manu-Tawiah and Martin, 1987). However, amino-acids composition is strain and substrate dependent but also function of culture conditions (Hadar et *al.*, 1992).

5.3.1.2 Polysaccharide-derived products (PS)

Major peaks derived from polysaccharides in fungus pyrolysate (Figure 5-2) are most likely issued from glucose polymers: furan, 2-methyl (PS28), 1,4:3,6-dianhydro-.alpha.-d-glucopyranose (PS21), Furan, 2,5-dimethyl- (PS35), 2-Cyclopenten-1-one, 2-hydroxy-3-methyl- (PS34) and 2-Cyclopenten-1-one, 2,3-dimethyl- (PS8). Those products can effectively be encountered in cellulose pyrolysates (Lu et *al.*, 2012; Moldoveanu, 2001). Some of them are also reported in other kind of glucose polymers, PS21 can notably be found in 1-3 branched glucan pyrolysates. Glucose polymers constitute an important part of fungi polysaccharides. They can be under the form of glycogen and trehalose (storage function) or hyphal linked glucans excreted by fungi (Gutierrez et *al.*, 1995; van der Kaaden et *al.*, 1984; Walker and White, 2005). PS28 can come from chitin (glucose-like polymer). The minor peak of 2-furancarboxaldehyde, 5-methyl- (PS9) can derive from glycogen (Kalač, 2009; Moldoveanu, 1998).

Finally, mannitol was also described as a major carbohydrate in fungi (Kalač, 2009; Obatake, 1998) but no mannitol specific products were found after pyrolysis of *P. brumalis* BRFM 985 mycelium. Thus, no conclusion about the amount of this polymer in *P. brumalis* BRFM 985 can be provided. The maltol (PS36) peak does not derive from mannitol (Ross et *al.*, 2009) but from glucose polymers since reported in cellulose pyrolysis (Moldoveanu, 2001).

5.3.1.3 Possible presence of small lignin amount

The presence of one small guaiacol (LIG1-G) peak in the pyrolyzate of the mycelium can be noticed. This compound is considered as a lignin marker (LIG) and, to our knowledge, no other origin are identified. The presence of small lignin amount in WRF that had grown on lignocellulosic substrate is very likely since compounds enter into the hyphae (Martínez et *al.*, 2005) and hyphal wall was labelled with anti-lignin antibodies (Burlat et *al.*, 1997). These mechanisms are unlikely with the mycelium of *P. brumalis* that grew on synthetic medium (without lignin).

However, high amount of dietary fiber can exist in fungi fruiting body. A part of this fiber is Klason lignin, as notably observed in white-rot (*Pleurotus spp*. and *Ganoderma lucidum*) but also for other kinds of fungi (Cheung, 1997; Ragunathan and Swaminathan, 2003). Klason lignin determination is based on acid precipitation and can notably be contaminated with proteins (Reeves and Galletti, 1993). Klason lignin (KL) measurement is the most common method to estimate lignin amount with Van Soest method. No one of these methods allow the obtaining of a pure analytical fraction without matter losses (Giger S., 1985). KL values may often be more accurate (Goff et *al.*, 2012; Hatfield et *al.*, 1994). Thus, the possibility to encounter lignin-like fractions rather than real lignin in fungi cannot be excluded. Further investigations are required to conclude.

5.3.2 Straws characterization

Pyrograms of untreated straw and straws pretreated in the selection step are presented in Figure 5-3. Given that three main constituents of wheat straw are cellulose, hemicelluloses (carbohydrates) and lignin (polyphenols) (Sun, 2010); straws pyrograms were dominated by lignin- and polysaccharide-derived compounds.

5.3.2.1 Lignin derived pyrolysis products

Lignin-derived pyrolysis products dominated pyrolysates of wheat straws, as already observed by Reeves and Galletti (1993). Lignin is formed by an association of three monolignols: p-hydroxyphenyl (H, no methoxy substituent), guaiacyl (G, methoxy substituent) and syringyl (S, di methoxy substituent) units. Pyrolysis products were labeled according to their origin: S- and G-units derived compounds are classified as LIG-S and LIG-G, respectively. Compounds derived from H-units in pyrograms are often considered as unspecific, especially because they can also originate from cinnamic acids (not lignin units) which are frequent in non-woody-plants (Camarero et *al.*, 1999; Ross and Mazza, 2011). In addition, H-units are less abundant in grasses compared to S- and G-units (Fahmi et *al.*, 2007). For those reasons, H-units were not considered in the current study.

Major peaks in straws pyrolysates (Figure 5-3) were guaiacol (LIG1-G), phenol, 2,6-dimethoxy- (LIG6-S) and 2-Methoxy-4-vinylphenol (UN13). Guaiacol (LIG1-G) and/or 2-Methoxy-4-vinylphenol (UN13) were already reported as major compounds in untreated wheat straw. Phenol, 2,6-dimethoxy- (LIG6-S) was not reported as a major peak in straws pyrolysates, even if generally present (Camarero et *al.*, 1994; Reeves and Galletti, 1993; Ross and Mazza, 2011). However, a major peak of a compound (4-Vinyl-2,6-dimethoxyphenol) with a chemical structure close to the one of LIG6-S was observed by Camarero et *al.* (1994).

2-Methoxy-4-vinylphenol (UN13) was classified as unspecific compound since it was identified in lignin samples but also in small amounts in xylan samples. However, lignin origin is favored (Dignac et *al.*, 2005) because lignin residues can be found in xylan fractions. Methyl guaiacol (LIG2-G) is additionally quite important in pyrolysates (Figure 5-3). Methyl guaiacol (LIG2-G) and vanillin (LIG22-G) were major

compounds of pyrolyzed milled wood lignin (Terrón et *al.*, 1995). Vanillin (LIG22-G) is a minor peak in studied pyrolysates.

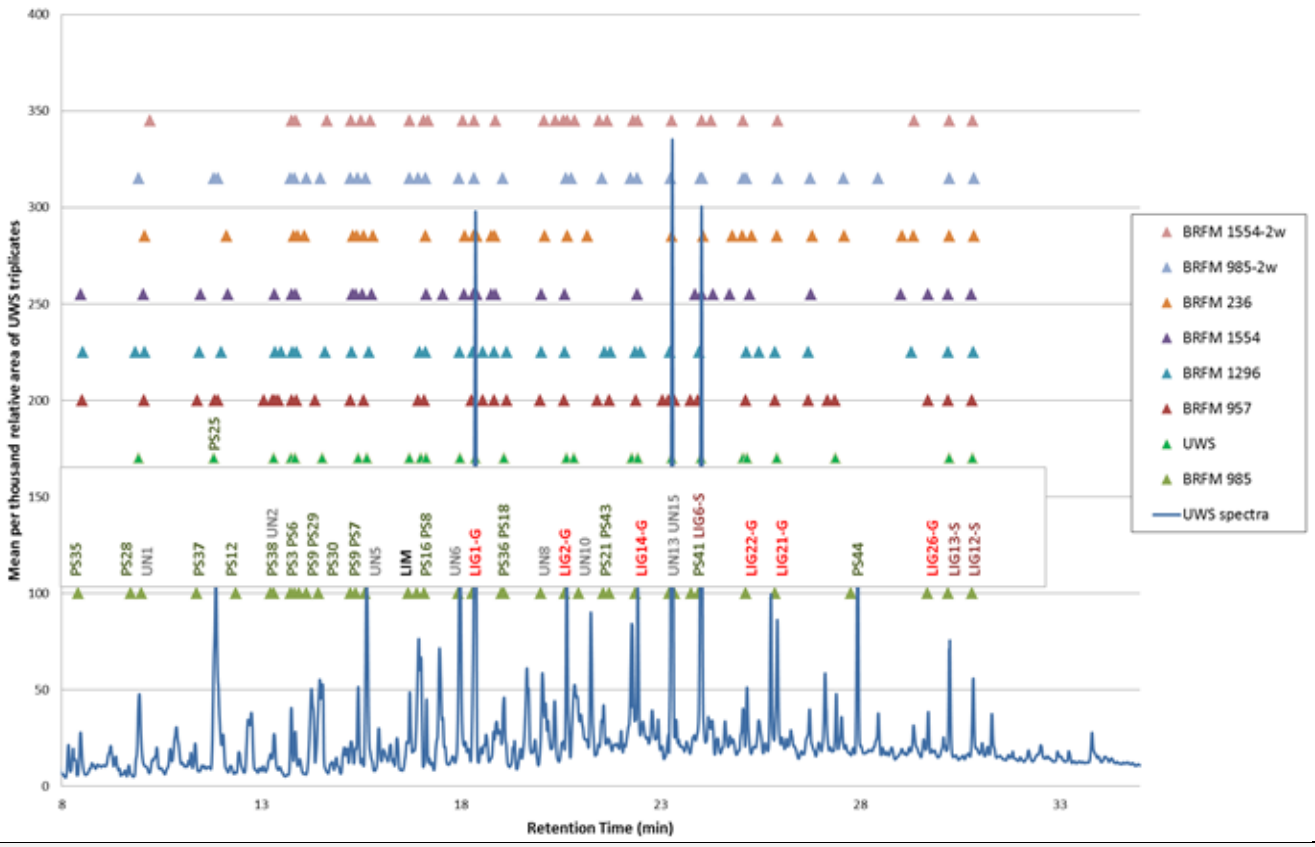


Figure 5-3. Pyrograms comparison of untreated wheat straw (UWS) and fungal treated straws.
Compounds codes are reported in Annex 8 and Annex 9.

Yield and composition of pyrolysates can vary notably owing to the variety of py-GC-MS operating conditions (heating rates, temperatures…) (Carlos Amen-Chen et *al.*, 2001). Moreover, lignocellulosic samples even within a same species can also vary in their composition (growth stage, tissue…).This explains why similarities and few variations were observed between the current study and literature on pyrolysis-GC-MS of wheat straw.

5.3.2.2 Polysaccharide-derived products

Cellulose is a polymer of glucose whereas wheat straw hemicelluloses are mainly composed of xylan with other carbohydrates in minor amount (glucose…). Therefore, polysaccharide-derived pyrolysis products were abundant in the pyrolysates of wheat straw. Major polysaccharide compounds encountered in straw samples are 1,2-Cyclopentanedione (PS30), 1,2-Cyclopentanedione, 3-methyl- (PS16) and furfural (PS25) (Figure 5-3). Furfural (PS25) was reported as a major compound by Ross and Mazza (2011) in herbaceous crop pyrolysates (including wheat straw). They also reported PS16 in wheat straw pyrolysates. Those compounds (PS30, PS16 and PS25) were not found in the mycelium pyrolysates (Figure 5-2) whereas some other compounds were both present in straw and fungus chromatograms such as furan, 2-methyl (PS28), 1,4:3,6-dianhydro-.alpha.-d-glucopyranose (PS21), furan, 2,5-dimethyl- (PS35). For PS-class compounds, it is sometimes possible to identify the carbohydrate origin (glucose, xylose…), e. g. 1,4:3,6-Dianhydro-

.alpha.-d-glucopyranose (PS21) derived from glucose polymers (Dignac et *al.*, 2005). Glucose is a major compound in fungus but also in straws (cellulose and hemicelluloses). Moreover, no specific compounds from hemicelluloses were identified in the current study. Thus, polysaccharides originating from fungus cannot be well distinguished from those derived from straws.

5.3.2.3 N-containing compounds

Only one N-containing compound was found (in sample 1554-2w). This N-containing compound, pyridine (N4), originated from protein or chitin. The low amount of N-class can be explained by the poor protein content of wheat straw (around 3%) (Lee, 1997) and also by the low amount of fungal biomass on pretreated straw in comparison with other straw compounds (qPCR results in Table 5-2).

5.4 Comparison between py-GC-MS data, other characterization technics and anaerobic digestibility

Cellulose and hemicelluloses, especially amorphous structure, are converted into methane during anaerobic digestion. Lignin, with its protective role, is recalcitrant to degradation (Sun, 2010). Anaerobic digestibility is most often measured by a BMP-test that consists to digest a substrate under optimal conditions. However, with slowly degradable substrates such as straw, several months can be required to reach the BMP value. The characterization of fungal pretreated wheat straws helps to evaluate the pretreatment efficiency and to understand modifications of BMP values. The possibility to use py-GC-MS to characterize fungal pretreated straw for anaerobic digestion is investigated. It relies on detection of major differences between untreated and pretreated straws (notably lignin content) and on the possibility to compare fungal treated straws between them in order to assess the efficiency of fungal pretreatment (PS/LIG…).

Pyrolysis peaks were integrated in order to compare the yield of pyrolysis products obtained in the treated and untreated samples. Peak areas were expressed in per thousand of total peak area per chromatogram. For each molecule, average relative areas per thousand for triplicates were calculated (Annex 9).

Identification results and relative areas per thousand for straw samples are reported in Annex 9 and ordered by their retention time (RT). Then, sums of average relative areas were calculated for chosen groups of compounds (PS, LIG-G, etc.). Lignin percent for pyrolysis is calculated by comparison of sum of relative areas for all compounds with sum of relative areas for lignin compounds. This **quantitative overview of main compounds in pyrolysates** is reported in Table 5-2. In this table, a **comparison with straw** composition (determined by acid hydrolysis with NREL method) and fungal biomass amount (determined by qPCR) is also presented.

Comparatively to untreated straw, it can be observed a tendency for PS compounds to increase in pyrolysates. It is likely that the increase is due to lignin removal that leads proportionally to more carbohydrates in the pyrograms (Ohra-aho et *al.*, 2013; Reeves and Galletti, 1993). Then, the amount of compounds with a lignin origin (LIG) is greater in untreated wheat straw (UWS). This is in accordance with Klason lignin measurements.

It is difficult to find a link between composition determined with NREL method (acid hydrolysis) and pyrolysis data. It does not exist linear correlations for lignin percentages ($R^2 = 0.07$, n = 8) or PS/LIG ratios obtained with the two measurement technics (Annex 10). In those conditions, supplementary information is required to compare samples between them based on their PS or lignin contents determined with py-GC-MS data. For PS/LIG ratios, the mismatch can be explained by the little efficiency of PS amount estimation with pyrolysis compared to lignin.

Carbohydrate polymers have a structure less stable than lignin and they fragment easily upon pyrolysis to produce non-diagnostic low molecular weight compounds (Ralph and Hatfield, 1991). Lignin measurement with pyrolysis can depend of several parameters but the most likely explanation for the mismatch between the two lignin content measurement methods is that small variations between samples imped the establishment of a significant correlation. The addition of the mycelium sample (lignin amount close to zero) improved the R^2 coefficient ($R^2 = 0.5565$, n = 9, Annex 10).

5.4.1 Variations of the S/G ratio

Concerning the S/G ratio determination with pyrolysis, no chemical measurements were carried out to compare results. Monomers coming from lignin **G-units (LIG-G) were more abundant than S-units** (LIG-S) in the pyrolyzates. The same observation was done by Fahmi et *al.* (2007) with grass lignins. S-units are, in general, more biodegradable than G-units (Camarero et *al.*, 2001; Monlau et *al.*, 2013), it also the general case for WRF degradation (Chapter 1, 1.6.1). In the current study, S/G measured with pyrolysis-GC-MS were between 0.5 and 0.6 in most samples (Table 5-2). Río et *al.* (2012) and Terrón et *al.* (1993) found similar results after pyrolysis-GC-MS of wheat straw. There is a good reproducibility since slight different analytical conditions were used (pyrolysis temperature, etc.) between studies.

The **S/G** ratios of lignin can be estimated by various methods, most usual are nitrobenzene oxidation (NBO), cupric oxidation and thioacidolysis (Camarero et *al.*, 1994; Hedges and Ertel, 1982; Lapierre, 2010). 13C Nuclear Magnetic Resonance (NMR) can also be used but seems not well adapted for S/G determination in wheat straw (Fidalgo et *al.*, 1993). Chemical methods break uncondensed bonds, mainly β-O-4 ether bonds that are the main linkages in lignin (Lapierre, 2010). By contrast, Py-GC-MS is able to break some C-C bounds (condensed) between lignin units (Ralph and Hatfield, 1991; Tsuge and Matsubara, 1985). G-units are more implied in condensed linkages than S-units (Lapierre, 2010; Monlau et *al.*, 2013). Consequently, G-units estimation with py-GC-MS increases compared to other methods, decreasing S/G ratio. S/G obtained with py-GC-MS may be closer of the actual value (Ross and Mazza, 2011). For wheat straw, S/G ratio reached 0.8 when measured with NBO or cupric oxidation (Sun et *al.*, 1995); 0.9 with thioacidolysis (Lapierre et *al.*, 1988). To obtain similar results between chemical and py-GC-MS determinations of S/G ratio, some specific pyrolysis compounds can be selected (Lima et *al.*, 2008; Nunes et *al.*, 2010) or alkaline lignin extraction must be carried on before py-GC-MS (Terrón et *al.*, 1993).

Table 5-2. Characterizations with py-GC-MS, acid hydrolysis and qPCR and BMP of fungal pretreated straws during the selection step.

S. D: Standard-deviation. N. A.: Not Available; [a], [b] S/G values followed by the same letter are not significantly different (ANOVA, α = 0.05); *exclusion of one value among three; [xx] High standard deviation, value not considered in PCA and in Figure 5-7.

	Samples		236	957	985	1048	1296	1554	985-2w	1554-2w	UWS
Pyrolysates	Sum for all compounds		452.23	487.22	482.40	433.70	448.67	418.33	615.07	564.03	696.84
	Sum for LIG		100.38	76.15	72.77	60.17	85.78	105.39	182.93	181.10	216.54
	Sum for PS		201.44	200.34	197.18	247.83	162.78	203.08	189.02	129.56	170.32
	Sum for LIG G		68.32	57.97	48.96	40.07	56.31	69.34	113.55	119.12	136.62
	Sum for LIG S		32.06	18.19	23.81	20.11	29.47	36.06	69.38	61.98	79.92
	PS/LIG		2.00	2.65	2.31*	7.81[xx]	1.90	2.01	1.04	0.72	0.78
	LIG S/LIG G		0.47[a]	0.31[b]	0.49[a]	0.96[xx]	0.52[a]	0.44*[xx]	0.61[a]	0.52[a]	0.59[a]
	Lignin (%)		22.20	15.63	15.08	13.87	19.12	25.19	29.74	32.11	31.07
	S. D. (% of the mean)	LIG	9.24	5.88	22.66	65.94	8.84	20.99	7.43	0.83	8.53
		PS	14.48	17.35	29.08	16.41	12.61	29.10	9.68	9.22	18.33
		LIG G	10.52	3.42	24.33	76.01	8.93	31.94	7.73	2.50	9.33
		LIG S	6.54	21.89	19.67	51.16	8.76	9.87	14.74	6.46	8.05
		PS/LIG	5.66	21.79	23.39*	111.38[xx]	10.39	37.99	15.53	9.65	11.46
		S/G	4.24	22.09	8.36	97.23[xx]	1.71	11.58*	15.94	8.90	5.17
Acid hydrolysis results (Klason lignin, etc.)	Cellulose (%TS)		39.39 ± 0.40	38.30 ± 0.53	41.05 ± 0.22	38.82 ± 0.44	36.40 ± 0.73	36.77 ± 0.41	39.45 ± 0.52	41.49 ± 0.74	N.A.
	Hemicelluloses (% TS)		28.90 ± 0.92	29.87 ± 0.27	29.34 ± 0.29	30.90 ± 1.03	30.48 ± 0.63	30.63 ± 1.20	31.95 ± 2.69	32.01 ± 1.71	N.A.
	Lignin Klason (% TS)		22.93 ± 0.02	18.08 ± 0.21	17.12 ± 0.10	18.31 ± 1.13	19.56 ± 0.79	18.59 ± 0.46	18.16 ± 0.17	21.03 ± 0.51	N.A.
	Sum cellulose, hemicelluloses (% PS)		68.29 ± 1.32	68.17 ± 0.79	70.39 ± 0.51	69.72 ± 1.47	66.88 ± 1.36	67.40 ± 1.60	71.40 ± 3.21	73.50 ± 2.45	N.A.
	PS/LIG		2.98 ± 0.06	3.77 ± 0.09	4.11± 0.05	3.81 ± 0.32	3.42 ± 0.21	3.63 ± 0.18	3.93 ± 0.21	3.50 ± 0.20	N.A.
qPCR	Fungal biomass (mg/g straw)		N.A.	73.1 ± 5.6	49.5 ± 2.8	48 ± 8.9	N.A.	19.8 ± 2.2	28 ± 6	28.3 ± 7.6	-
Anaerobic digestibility	BMP (NmL/g VS)		217 ± 43	266 ± 23	280 ± 5	232 ± 2	243 ± 3	212 ± 2	N.A.	N.A.	190 ± 6

In the current study (Table 5-2), most samples have a similar S/G ratios, it may show no preferential consumption for G-units or for S-units in the tested conditions of fungal pretreatments. In contrast, the sample treated with BRFM 957 has a lower S/G ratio (S/G = 0.31) than the one of untreated samples (S/G = 0.59). This suggests an increased consumption of S-units compared to G-units for BRFM 957. Straw pretreated with BRFM 957 was extensively degraded (high masses losses, as discussed in Chapter 7, 7.3). This degradation explains the particularly low S/G and concerns both carbohydrates and lignin as it can be observed with acid hydrolysis results. In literature, Substrate, strains and culture conditions influence remaining S/G ratio in substrate (Chapter 1, 1.6.1). Backa et *al.* (2001) suggested that fungi may first degrade S-units. By contrast, Agosin et *al.* (1985) observed a consumption of G units before S units by WRF. In the current study, similar S/G ratios between samples pretreated two weeks or three weeks did not allow concluding on this topic.

5.4.2 PCA to study relationships between samples characteristics

A Principal Component Analysis (PCA) was built to investigate relationships between BMP, variables measured with py-GC-MS and with other methods (acid hydrolysis, qPCR…). Moreover, this PCA would allow identifying supplementary compounds of interest in pyrolysates since small peaks are frequently important in making discriminations between samples (Wampler, 1999). To increase samples variability and so to obtain a more robust statistical analysis, the mycelium sample was added to straws samples (Table 5-2 and Table 5-3) to carry out the PCA. Due to numerous missing values, only pyrolysis compounds which had a value of relative area for more than the half of the samples were taken into account. Several variables influencing the BMP were studied: fungal biomass determined by qPCR (qPCR on PCA, expressed in mg/g straw), Klason lignin (LIG), hemicelluloses (HEMI) and cellulose (CEL) determined by NREL method (expressed in % TS) and the resulting PS/LIG (PS/LIG NREL) and finally, PS/LIG obtained with py-GC-MS (PS/LIG-pyr). BMP was also included as variables to build the PCA (expressed in NmL/g VS).

On three axes, PCA contains 75% of the whole information contained in data. The plan defined by axis one and two (plan 1-2, Figure 5-4) and the one defined by axis one and three (plan 1-3, Figure 5-5) will be further described; except for variables that are not well represented in the plans (both coordinates close to or inferior to 0.5). Plan 2-3 is not presented as no additional information can be obtained from this plan. Samples are well represented according to the criteria $Dmod_X$ (see 5.2.4.2).

5.4.2.1 qPCR variable opposed to straw composition variables in the plan 1-2

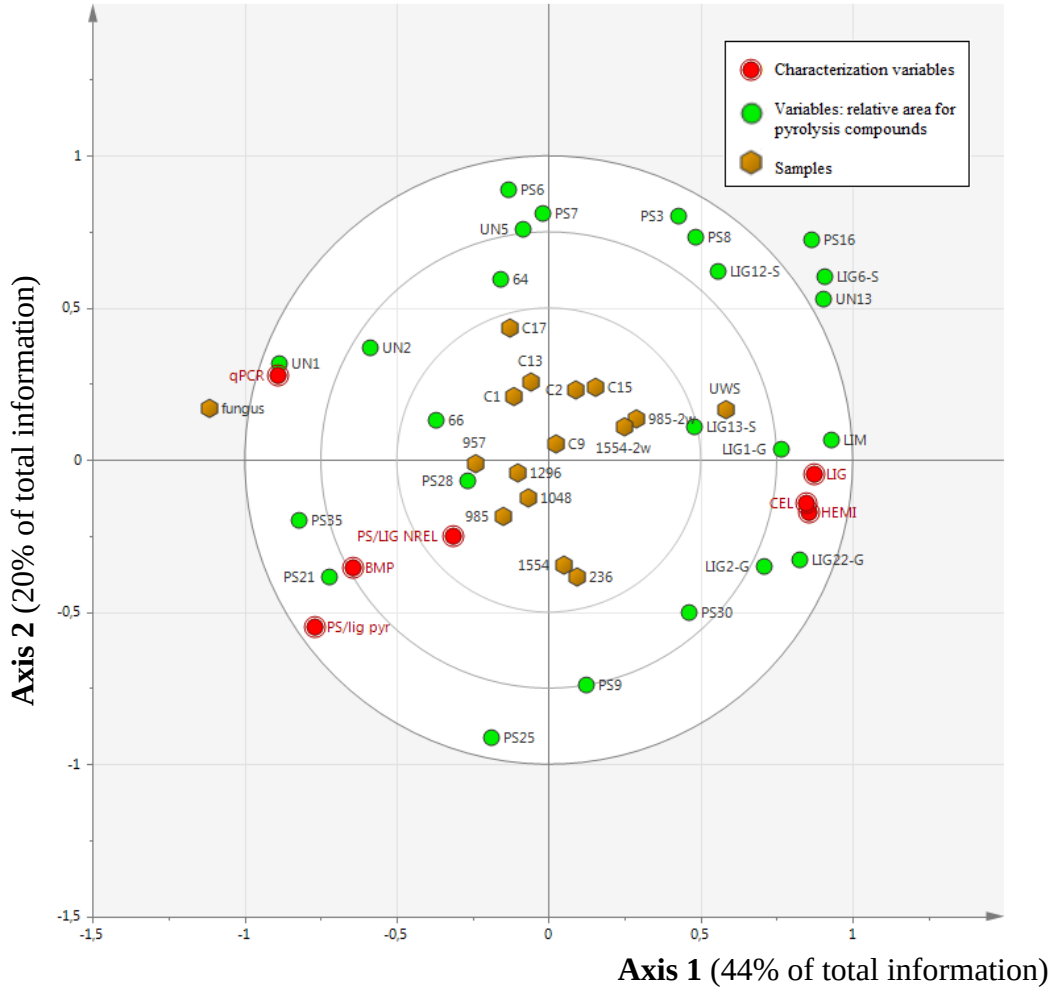


Figure 5-4. Plan 1-2 of the PCA with pyrolysis and other characterization variables.

In plan 1-2, axis 1 is defined by qPCR variable (left) that is opposed to the content of LIG, HEMI and CEL (right). This opposition is explained because maximal amounts of LIG, CEL and HEMI correspond to a minimal amount of qPCR variable (equals to zero for UWS sample) and vice versa (CEL, HEMI and LIG equals to zero for fungus sample).

There is a correlation between PS/LIG-pyr and BMP. This observation suggests that py-GC-MS may be used as pre-selecting technic in anaerobic digestion studies (almost on wheat straw). BMP variable is opposed to the recalcitrant LIG. Surprisingly, BMP is opposed and poorly linked to CEL and HEMI that constitute the substrate for anaerobic digestion. It may be due to little variation between samples for HEMI and CEL content (Table 5-2). qPCR variable is positively and poorly linked to BMP on the plan 1-2.

Concerning pyrolysate compounds, when LIG6-S, LIG1-G, and UN13 are high, lignin amount is also high, as expected (major peaks in straws pyrolysates). The control sample UWS is the one having the highest lignin content. It is, thus, the closest sample to lignin-related variables. PS16, also major in straw pyrolysates, is high when HEMI and CEL are high. PS21 and PS35 are anti-correlated to HEMI and CEL and correlated to qPCR variable. In this study, they may represent more fungal biomass than straw: they are

major peaks of fungus pyrolysate and minor ones on straws pyrolysates. UN1 and UN2 are correlated to qPCR variable since they are major peaks in fungus pyrolysate.

5.4.2.2 Correlation between BMP and PS/LIG-pyr or PS/LIG NREL in the plan 1-3

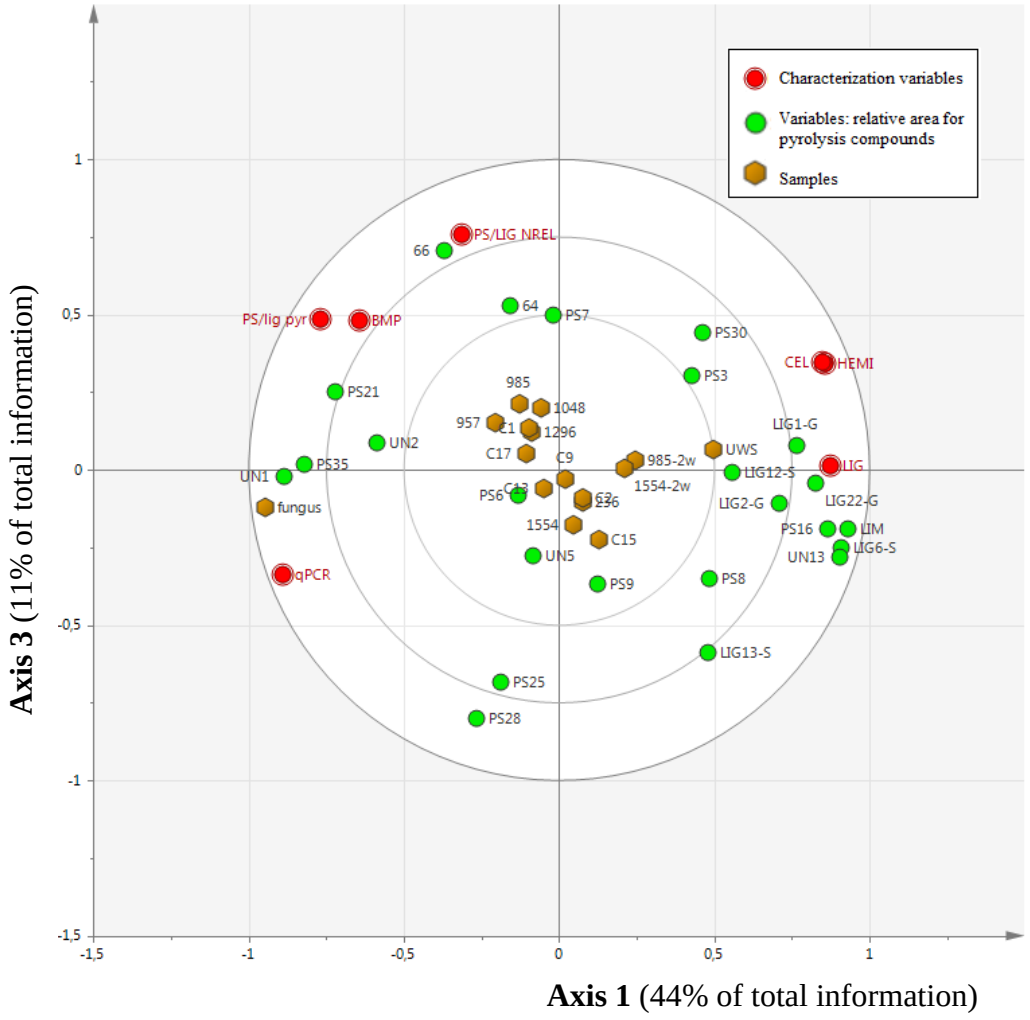


Figure 5-5. Plan 1-3 of the PCA with pyrolysis and other characterization variables.

On plan 1-3 (Figure 5-5), PS/LIG NREL is well represented compared to the projection in plan 1-2. On plan 1-3, PS/LIG NREL is slightly positively correlated to PS/LIG pyr and BMP. PS/LIG pyr and BMP are highly correlated as in the plan 1-2. This relationship suggests that py-GC-MS would allow classifying pretreated wheat straws according to their anaerobic digestibility. As previously observed, PS35, PS21, UN1 and UN2 were correlated to fungus sample and qPCR variable; BMP is almost independent from qPCR variable, CEL and HEMI.

Table 5-3. Pyrolysis and characterization data used for PCA for samples from the optimization step.
[xx] High standard deviation, value not considered in PCA and in Figure 5-7.

		C17	C15	C13	C9	C2	C1	fungus
Average relative areas per thousand for triplicates	LIG1-G	14.02	22.94	16.24	24.61	21.98	17.34	4.92
	LIG22-G	3.18	10.82	4.99	10.38	8.28	6.43	
	LIG2-G	2.81	5.77	2.72	2.43	4.56	2.15	
	LIM	1.43	3.86	1.90	2.28	3.36	1.92	
	PS21	3.64	1.69	1.48	4.69	4.79	4.94	23.73
	PS28							18.81
	PS3							3.97
	PS30	6.00	6.31	4.53	15.01	9.67	8.43	
	PS35	2.03	0.00	2.28	0.00	1.07	5.80	16.58
	PS42							3.04
	PS46							3.03
	PS6							5.76
	PS7							8.85
	PS8							5.00
	PS9						2.29	6.10
	UN1	23.93	13.50	19.10	12.96	14.49	22.75	58.11
	UN2	17.79	8.83	9.03	10.99	9.90	9.52	13.29
	UN5							28.84
	UN6							39.68
	64	6.06	3.05	4.80	3.62	4.18	4.83	
	66	11.42	7.59	8.46	7.58	9.21	11.89	11.33
Main characteristics	LIG (% TS)	21.8 ± 0.1	22.5 ± 0.7	20.1 ± 0.2	19.0 ± 0.2	22.5 ± 0.1	17.6 ± 0.3	0.00
	HEMI (%TS)	28.9 ± 1.0	25.5 ± 0.9	27.7 ± 0.9	27.9 ± 0.0	31.1 ± 0.9	31.4 ± 1.9	0.00
	CEL (%TS)	39.2 ± 0.7	34.9 ± 0.5	39.4 ± 0.6	37.8 ± 0.7	35.2 ± 0.8	38.5 ± 1.1	0.00
	qPCR variable (mg/g straw)	N.A.	N.A.	N.A.	48.1	38.38	63.4	1000.00
	PS/LIG nrel	3.1 ± 0.1	2.7 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	2.9 ± 0.1	4.0 ± 0.2	
	PS/LIG pyr	1.47 ± 0.32	0.78 ± 0.11	2.64 ± 1.66 [xx]	1.51 ± 0.07	1.29 ± 0.37	2.01 ± 1.01[xx]	-
	BMP	194 ± 13	176 ± 12	205 ± 17	268 ± 16	183 ± 3	247 ± 14	281±31

5.4.3 Fungal biomass (qPCR variable)

Fungal biomass determination with qPCR was not carried out for the sole brown-rot fungus of this study (BRFM 236). As explained previously (in 5.3.1.1), high amount of phenylalanine in the mycelium of BRFM 985 is suspected because of high peaks of UN1, UN2 and small one of 64 on fungus pyrolysate. UN1 and UN2 relative peak areas for fungal pretreated samples are well correlated to fungus samples and qPCR variable (plan 1-2 and 1-3). They may be good indicators of fungal development as they are correlated to fungal biomass, whether separately (R^2=0. 72 for UN1 and R^2=0.68for UN2, Annex 11) or in addition with compound 64 (R^2=0.81, n=8, Figure 5-6). UN1 and UN2 seem preferable to study fungal biomass than PS35 and PS21 also correlated to qPCR variable (plan 1-2 and 1-3) because carbohydrates are less stable during pyrolysis. Thus, pyrolysis would allow monitoring WRF growth during pretreatment of wheat straw.

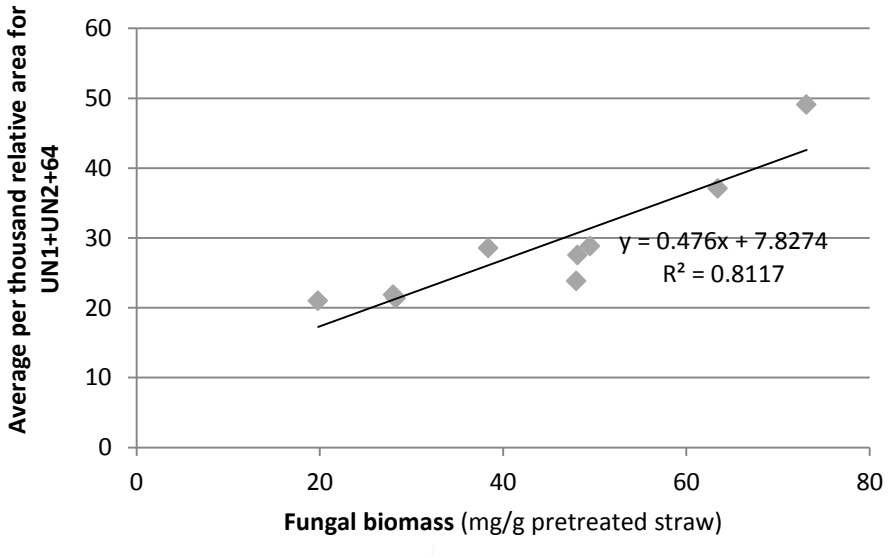


Figure 5-6. Correlation between Toluene (UN1), Styrene (UN2) and ethylbenzene (64) (Py-GC-MS) and fungal biomass (qPCR) for straws pretreated with WRF.

To finish, in the pyrogram of the stone deteriorating fungi *Ulocladium atrum* (not WRF), diverse compounds found in *P. brumalis* pyrolysate were also in high amount: toluene (UN1), methylfuran (PS28), alpha-d-glucopyranose (PS21), dimethylfuran (PS35). Styrene (UN2) and phenol (UN5) were also in moderate amount (Gutierrez et *al.*, 1995). Thereby, some properties of *P. brumalis* pyrolysate might also be valid for other fungal groups. High presence of toluene (UN1) and styrene (UN2) in mycelium pyrolysate of non WRF is encouraging to investigate the possibility to quantify fungal biomass amount with py-GC-MS for other kind of fungi, such as BRF.

5.4.4 Polysaccharides (PS)/Lignin (LIG) ratio determined by py-GC-MS

Whereas no linear correlation exists between PS/LIG-pyr and PS/LIG determined with NREL method ((hemicelluloses + cellulose)/lignin Klason) (Annex 10). A correlation between BMP and PS/LIG-pyr or PS/LIG NREL was revealed by the PCA (plan1-3). This correlation was drawn (Figure 5-7). Samples with particularly high standard-deviations (>50% of the triplicate average) for PS/LIG pyr (C1, C2 and 1048 in Table 5-2and Table 5-3) were excluded from the plotted correlation (Figure 5-7).

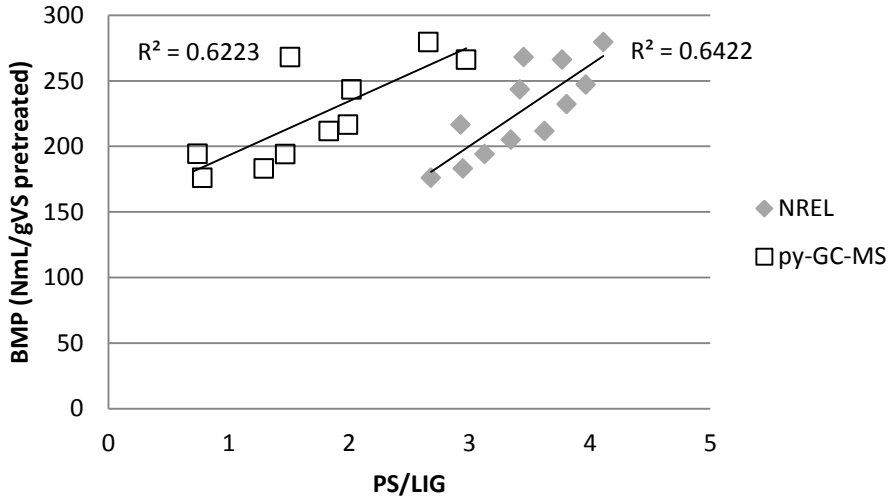


Figure 5-7. Correlation between PS/LIG determined with Py-GC-MS or NREL method and BMP.

Correlations from Figure 5-7 highlight the interest of PS/LIG ratio to compare fungal pretreated straws for anaerobic digestion application and the possibility to use pyrolysis to investigate pretreatment efficiency thanks to PS/LIG pyr.

Since different culture conditions were used in this study, it seems possible to use PS/LIG pyr to access anaerobic degradability of wheat straw samples obtained with slight different fungal pretreatments (such as culture temperature varying between 25-30°C or culture duration between 12.5 and 21days). Notwithstanding, it cannot be excluded that classification of fungal treated samples based on their PS/LIG pyr ratio would be more precise using the same pretreatment condition. Major compounds released and information they contain can be variable because not only dependent on analytical conditions (especially pyrolysis temperature and chromatographic separation conditions) but also on fungal culture parameters (strain, substrate, aeration, moisture…) (Carlos Amen-Chen et *al.*, 2001; Wan and Li, 2012). Finally, it seems possible to compare straws pretreated with different WRF strains with py-GC-MS to access pretreatment efficiency. The comparison might also include Brown-Rot Fungi (BRF) but more data are required to conclude (only one BRF strain tested in this study, *G. trabeum* BRFM 236).

5.5 Conclusion

Interestingly, a correlation was found between fungal biomass amount determined by qPCR and peaks areas of few compounds released by py-GC-MS of pretreated wheat straws. These compounds were notably toluene and styrene, present in high amount in mycelium pyrolysate. Thus, py-GC-MS allows the estimation of WRF biomass amount on pretreated wheat straws.

Anaerobic digestibility of fungal pretreated wheat straws can be accessed by the determination of the PS/LIG ratio with py-GC-MS. Slight variations in fungal pretreatments did not seem to alter the relationship between BMP and PS/LIG determined with py-GC-MS. However, PS/LIG ratios measured with py-GC-MS were not related to PS/LIG determined with usual chemical method (acid hydrolysis). Thus, py-GC-MS would be useful only to pre-select strains for fungal pretreatment of wheat straw for anaerobic digestion.

Chapter 6.

Solid-State Anaerobic Digestion of wheat straw: impact of substrate/inoculum ratio and of fungal pretreatment

Objectives

- To study the impact of S/I ratio in batch SSAD of wheat straw in order to obtain an efficient start-up phase
- To investigate the fungal pretreated substrate behavior in SSAD reactors with leachate recycle
- To characterize and to explain mechanisms observed during SSAD

Table of content

Highlights

- Requirement of S/I ratio between 2-3 (VS basis) to digest wheat straw in batch SSAD reactors with leachate recycle
- Capacity to recover from acidification for batch SSAD process having a TVFA/alkalinity ratio < 2 and VFA peak concentrations < 10 g/L
- Fungal pretreatment at pilot-scale with inoculation in non-sterile conditions
- Improvement of biodegradability (/g VS pretreated) with inefficient pretreatment in SSAD reactor
- Facilitation of start-up phase for SSAD of fungal pretreated straw leading to good methane production
- Necessity to optimize pretreatment to improve global efficiency of methane production (/g TS initial)

6.1 Introduction

High substrate loadings allow a higher methane production per reactor but also increase the risk of imbalance and acidification. A sufficient amount of methanogens that convert VFA into methane must be provided to avoid VFA accumulation during the start-up phase. Otherwise, anaerobic digestion can be inhibited. Thus, the determination of the optimum Substrate (S)/Inoculum (I) ratio for SSAD of a given substrate is of first interest.

The goals of this chapter were: (1) to investigate S/I ratio for batch SSAD of wheat straw during experiment I; (2) to investigate SSAD of pretreated wheat straw with *P. brumalis* BRFM 985 with an appropriate S/I ratio with experiment II.

The experimental design retained for reactors was one stage batch with leachate recycle. Indeed, batches require less capital cost and are relatively simpler (Li et *al.*, 2011). For solid substrate with low degradability and/or C/N ratio higher than 15, one stage processes are recommended (Kusch et *al.*, 2008; Weiland, 1993). Leachate recycle favors homogenization which facilitates the complete degradation of the substrate (Brummeler et *al.*, 1992). Continuous watering bears the risk to spread acidification during process initiation whereas discontinuous leachate recycle is assumed to expand methanogenic areas (Kusch et *al.*, 2012).

6.2 Material and methods specific to SSAD in batch reactors (Chapter 6)

6.2.1 Wheat straw pretreatment in a pilot-reactor

To inoculate wheat straw, *Polyporus brumalis* BRFM 985 was cultivated on pellets of miscanthus (Terr'nova ®: 48% cellulose, 27% hemicelluloses, 24% lignin). Those pellets were inoculated with mycelium from a sterile liquid culture (see 2.1.3). The mycelium was harvested, mixed with 25 mL sterile mQ water and ground one minute with Ultra-Turrax T25 Blender at 9500 rpm. Then, 50 g autoclaved miscanthus pellets were inoculated with a solution composed of 10 mL of previous crushed mycelium and 40 mL sterile mQ water. Miscanthus pellets were incubated for 24 h at 30°C before the addition of 25 mL sterile mQ water. Culture on miscanthus lasted from 7 to 10 days. Flasks, closed with cotton plugs, were manually agitated every day to favor the colonization.

One Roux flasks of colonized miscanthus pellets were used to inoculate 200 g of sterile straw. Straw was autoclaved in a bag with around 113 mL of mQ water/100 g straw. Then, 255 mL sterile mQ water/100 g straw and a metal solution filtered at 0.2 µm were added to the straw under sterile conditions: 5 mL of $CuSO_4$ and $FeSO_4$ at 18 mM for 100 g of straw. Bags were manually agitated to ensure a good repartition of fungal inoculum. Finally, seeded straw was putted in a 40 L aerobic reactor (Figure 6-1) in clean conditions. Before inoculation, the reactor was washed with a kärcher during 20 min at 120°C. The aerobic reactor contained two trays, the first one received 200 g of straw with a size of around 10 cm and the second one

received cut straw with scissors (1-5 cm). Fungal cultivation on straw lasted 13 days in the aerated reactor under high moisture content (≈90%). Pretreated straw was freeze-dried before further utilization.

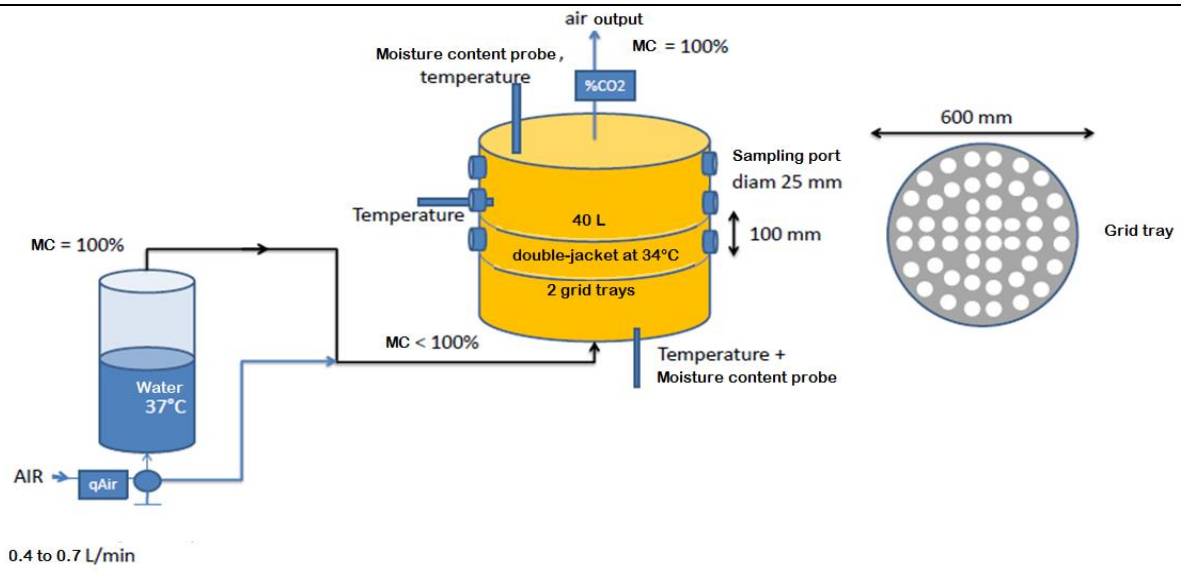


Figure 6-1. Aerobic pilot-reactor design for wheat straw pretreatment with *Polyporus brumalis*, BRFM 985.
MC: Moisture Content.

6.2.2 Solid state Anerobic Digestion in batch

6.2.2.1 SSAD reactors design

Experiments were performed using four 6-L glass reactors (head-space≈1L, leachate tank≈1.5L), water-jacketed to 37 °C (Figure 6-2). Leachate was collected in a liquid-phase reservoir at the base of reactors. Liquid pumps were set with timers to sprinkle the whole leachate volume over the biomass bed automatically every 2 hours. Biogas production was continuously measured using a mass flowmeter (Milli Gas counter-1V3.0 PMMA, Ritter Inc., Germany). Gas flow rate was acquired every 2 min by a computer.

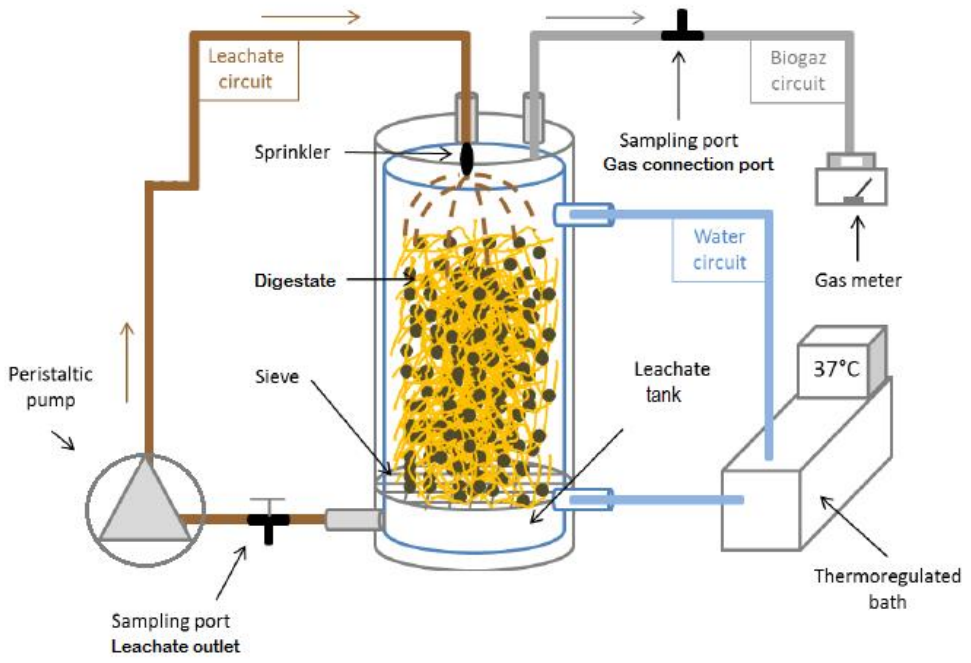


Figure 6-2. Schematic diagram of laboratory-scale SSAD digesters.

6.2.2.2 Experiment I: tests of several S/I

6.2.2.2.1 Inoculum

Inoculum used in this experiment came from a stable full-scale SSAD process fed with bull manure (containing high proportion of straw). The plant operates in batch at 41°C with leachate recycle and reaction duration of two months. To reduce the inoculum methane production, 1.7 kg was let for one month in 6-L batch reactors with 1.5 L of tap water in duplicates. A part of these leachates were used as liquid inoculum for Experiment I (nutrients, buffering capacity…). TS and VS of the inoculum are reported in Table 6-1.

6.2.2.2.2 Experimental configuration

Four reactors were carried on in parallel with different S/I ratios (VS basis): 1, 2, 4 and 11 (Table 6-2). Wheat straw and solid inoculum were mixed manually in a bag, transferred in a reactor and pressed one minute with 8 kg to mimic the compaction occurring in plants. Then, total liquid (tap water and liquid inoculum) were buffered with $NaHCO_3$ to the medium concentration of 1.3 g/L. TS content was between 15 and 17% within reactors. Head spaces were flushed with nitrogen gas. Measurements were stopped after the VFA peak.

6.2.3 Experiment II: fungal pretreatment for SSAD

6.2.3.1 Substrates and inoculum

Colonized miscanthus with *Polyporus brumalis* BRFM 985 was prepared in the same way as for the inoculation of wheat straw (see 6.2.1). Pretreated straw corresponded to the tray with cut straw (1-5 cm) of the pilot-reactor. To better compare all reactors and as pretreated straw was freeze-dried, untreated wheat straw used in Experiment II was cut (1-5 cm), autoclaved and freeze-dried. Uncolonized miscanthus pellets were also autoclaved and freeze-dried. After a sufficient duration, leachates of Experiment I did not contain VFA. They were used as liquid inoculum for Experiment II. Solid inoculum was constituted by a mixture between reactors with S/I = 1 and 2 of experiment I after two months of SSAD and a part of starved inoculum prepared in the same way as for experiment I. Substrates and inoculum TS and VS are reported in Table 6-1.

6.2.3.2 Experimental configuration

Three reactors were used to investigate the SSAD of pretreated wheat straw (Table 6-2). As pretreated straw contained colonized miscanthus, two reactors were prepared to measure the influence of miscanthus on SSAD. The first one contained raw straw and raw miscanthus (reactor MWS), and the second one raw straw and colonized miscanthus (reactor cMWS). The amount of miscanthus per reactor was representative of the proportion used for straw pretreatment. Proportion of straw in reactor MWS was chosen to obtain the same VS amount between reactor MWS and reactor with fungal pretreated straw (reactor FWS). Straw amount was the same between cMWS and MWS but substrate VS (miscanthus and straw) was slightly lower in

cMWS than in MWS. Finally, another reactor was prepared to estimate the endogenous production of the inoculum, it contained only inoculum and liquid. Amount of inoculum in this reactor was higher than for reactors with substrate (Table 6-2) in order to have the same %TS content in each reactor. Added tap water was buffered with $NaHCO_3$ to the average concentration of 1.3 g/L.

6.2.4 Analytical methods

6.2.4.1 SSAD monitoring parameters: leachate pH, VFA, alkalinity and biogas composition

On leachate samples, pH and VFA were measured regularly (daily at the beginning, weekly at the end). After a centrifugation step (20 min at 13 400 rpm in a micro-spin), VFA concentrations were quantified by gas chromatography using a Clarus GC 580 (PerkinElmer, USA) equipped with an auto-sampler and coupled to flame ionization detection (250°C) with H_2 and air as burning gas. Injector temperature was 220°C. Elite FFAP (PerkinElmer, USA) column (15 m long, 0.53 mm i.d., 1 µm thickness) was used with nitrogen (Nitrogen gas 5.0) as carrier gas at a flow of 7 mL/min. The GC oven temperature was programmed to increase from 80 to 120°C (hold time 6.5 min) and from 120°C to 140°C (hold time 3 min). Acid acetic equivalents of VFA used to calculate the ratio TVFA/alkalinity were: 1.0 (C_2), 0.818 (C_3), 0.682 (C_4), 0.588 (C_5) and 0.515 (C_6). These coefficients correspond to the ratio between molar mass of acid acetic and of the concerned VFA. Some alkalinity measurements were realized on leachate with pH titration using 0.1M HCl to endpoint of 4.3 (Ripley et *al.*, 1986).

Biogas composition in the head space was also analyzed with a Clarus GC 480 at the same frequency than leachate sampling and in the same way as already described for BMP assays (see 2.1.5).

6.2.4.2 BMP-tests

BMP of miscanthus and colonized miscanthus were measured in the same BMP-tests series whereas wheat straw and pretreated wheat straws were measured in another BMP-tests series. All samples were freeze-dried and ground to 1 mm (using Retsch® SM100). Concentration of the anaerobic inoculum in flasks was 5 g VS/L. Biogas composition was followed with Clarus GC 480 (PerkinElmer, USA).

6.2.4.3 Total Kjeldahl Nitrogen (TKN)

Kjeldahl nitrogen (TKN) was titrated using a Buchi 370-K distillater/titrator after mineralization of samples with Buchi digestion unit K438. A positive control, with known nitrogen concentration, and a blank were also measured.

Samples mineralization transformed sample nitrogen to ammonium in presence of H_2SO_4 (96%) and a catalyzer (Copper selenite). The color change of the sample solution from dark to clear or colorless meant a total mineralization of the sample. This process lasted from three to five hours depending on samples. The second step was performed with a distillater/titrator equipped with an auto-sampling system. Ammonium is converted to ammonia by adding sodium hydroxide (32%). Ammonia was then trapped into a boric acid solution (4%, at pH 4.65), to be converted to ammonium: $NH_4^+ + OH^- \rightleftharpoons NH_3 + H_2O$. The weak acid boric does not react with formed hydroxide ions that were titrated with the strong HCl acid (0.02 N).

6.2.4.4 Ammonium concentration in final leachate

Ammonium (NH_4^+) concentrations were determined with an ion chromatography system (ICS 3000 Dionex, USA) equipped with two pre-columns (NG1-2mm and CG16-2mm) and a separation column CS16-3mm. The detection was done by conductivity after the passage in a Cation Self-Regenerating Suppressor (CSRS-300-2mm). Eluent was hydroxymethanesulfonic acid (HMSA) with a concentration gradient between 25 and 40 mM and a flow rate of 0.3 mL min^{-1}.

6.2.4.5 Final Total Carbon (TC) in digestate

TC was measured as indicated in 3.2.2.1 with a carbon analyzer (TOC-V CSN, Shimadzu and Solid Sample Module-5000A).

6.2.4.6 Analysis of Variances (ANOVA)

As reported in 5.2.4.1, ANOVA, $\alpha = 0.1$, was realized with R software (version 3.2.1).

Table 6-1. Characteristics of substrates and inocula for Experiment I and II.								
	Experiment I			Experiment II				
	TS (% wet w)	**VS** (% TS)	**VS** (% wet w)	**TS** (% wet w)	**VS** (% TS)	**VS** (% wet w)	**BMP** (NmL/g VS)	**TKN** (%TS)
Wheat straw	91.1	95.2	86.7	97.3	94.2	88.1	247± 8	0.504
Fungal pretreated wheat straw				96.1	94.6	91	222 ± 8	0.765
Solid inoculum	13.7	76.3	10.5	11.7	73.6	8.6		
Liquid inoculum	2.1	51.5	1.1	1.4	43.2	0.6		
Miscanthus pellets				96.5	97.2	93.8	197 ± 4	0.128
Colonized miscanthus				36.7	96.5	35.4	185 ± 3	0.164

Table 6-2. Experimental set-up for Experiment I and II.								
		Experiment I				Experiment II		
						Solid inoculum	Miscanthus + Straw (**MWS**)	Colonized Miscanthus + Straw (**cMWS**)	Fungal pretreated straw (**FWS**)
S/I (VS basis)		**1**	**2**	**4**	**11**		3	3	3
Solid inoculum	(g wet w)	1015	750	475	205	1695	630	630	630
	(g VS)	106.4	78.8	49.7	21.3	146	54	54	54
Wheat straw	(g wet w)	155	200	240	280	0	155	155	195
	(g VS)	130	168	202	236		141	141	178
Miscanthus	(g wet w)					0	39.2	83.5	0
	(g VS)						36.7	29.7	
%TS of water saturated substrate		15.5	16.3	16.8	17.3	17.7	18.4	18.3	17.9
Total added liquid		2180	2350	2535	2720	1480	2360	2360	2360
including liquid inoculum	(mL)	600	600	600	600	1200	1200	1200	1200

6.3 Experiment I: tests of several S/I

6.3.1 Anaerobic digestion of wheat straw: start-up phase progress

The start-up period of SSAD batch is considered as the most critical (Brown and Li, 2013) that is why only the start-up phase was studied to choose the adequate S/I in Experiment I. Some lignocellulosic residues are rich in soluble compounds, e. g. wheat straw contains 12% soluble compounds (Sun, 2010). Those compounds are quickly converted to VFA. If methanogens are not in sufficient amount in the inoculum to convert those VFA into methane, a VFA accumulation can occur because methanogens growth is quite slow (Vavilin and Angelidaki, 2005). This accumulation can lead to detrimental pH drop that can sometimes stop anaerobic digestion (inhibition or death of methanogens).

For reactors with S/I of 1 and 2 (VS basis), cumulated methane yield always increased (Figure 6-3A). Methane yield is expressed per amount of total VS ($VS_{substrate}$ + $VS_{inoculum}$) since this quantity is approximately the same between reactors and because endogenous production from inoculum was not monitored. VS from the inoculum were quite stable and little degraded during Experiment I whereas VS from the substrate were extensively degraded. For S/I=1 and S/I=2, pH stayed near the neutrality during all start-up phase (Figure 6-3B). There was a production peak in daily methane production that reached almost 8 NmL/g VS_{total} (Figure 6-3D). These elements correspond to healthy reactors.

Notwithstanding, for S/I=2, the smallest pH was 6.5 on day 5 at the VFA peak (Figure 6-3C) and it corresponded to a decrease in daily methane production (Figure 6-3D). The peak in daily methane production occurring at day 9 was delayed compared to the one of S/I=1 occurring at day 4. This peak was concomitant to VFA peak for S/I=1 whereas it happened after the VFA peak for S/I=2 showing the necessity for methanogens to adapt. VFA peak reached 5 g/L for S/I=1 and 7 g/L for S/I=2 due to highest substrate amount (Figure 6-3C). Methane yield was a bit lower for S/I=2 than for S/I=1 (Figure 6-3A), even if values between these reactors were close (difference ≈ 10 NmL/g VS_{total} for the last measurement at day 15). However, values were identical until day 4, when pH was close to 6.5 for S/I=2 and where daily methane yield slightly decreased to increase again with pH (day 6-7). Moreover, VFA peak was large and their concentrations were almost identical between day 4 and 8 showing that this reactor had been close to instability (Figure 6-3).

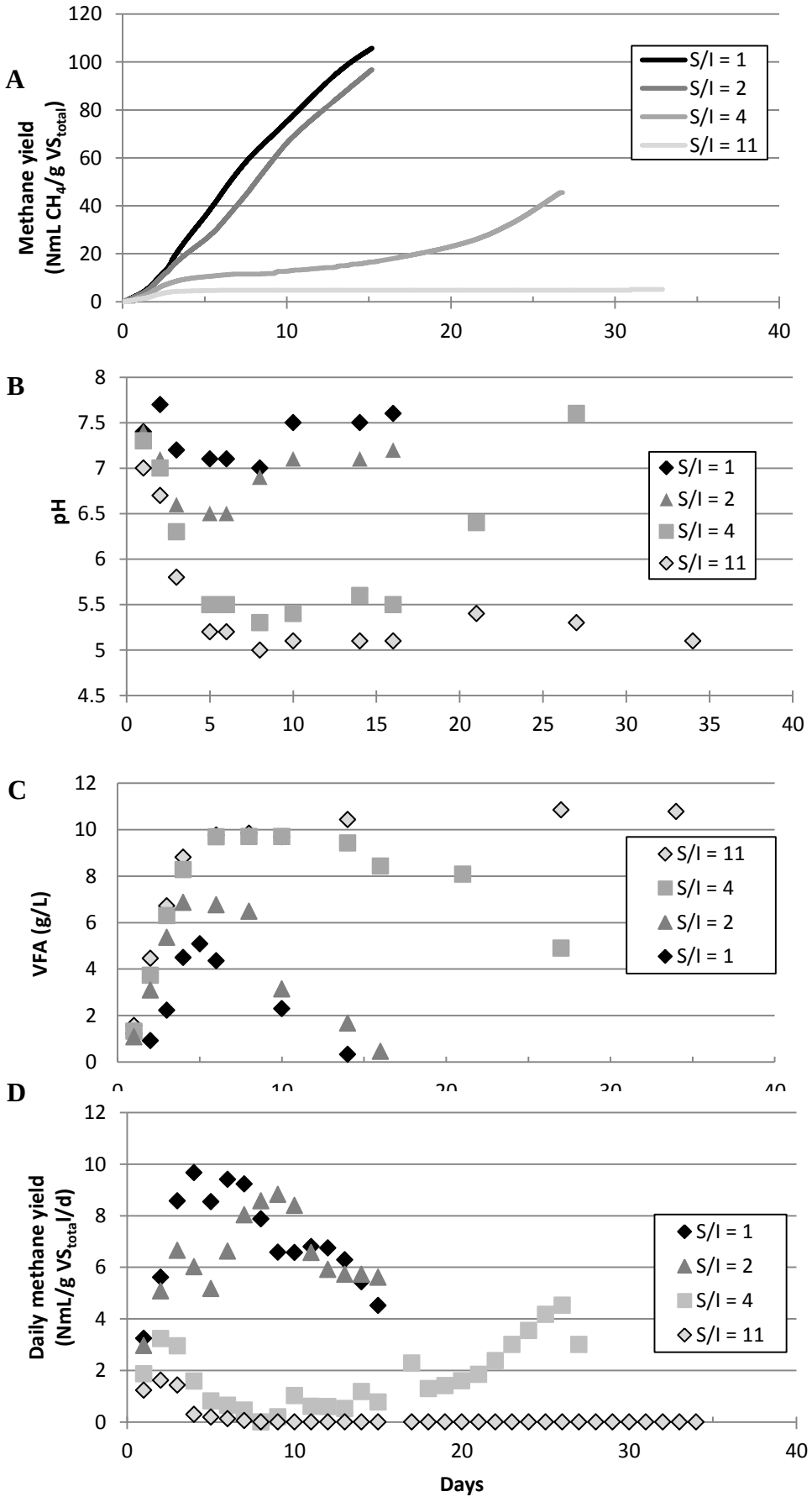


Figure 6-3. Main parameters of anaerobic digestion in function of time for Experiment I testing several S/I ratio.

A. Methane yield. B. Leachate pH. C. Leachate global VFA concentration. D. Daily methane production.

With S/I = 4, acidosis occurred during almost ten days (pH ≈ 5.5 from day 5 to 15). Daily methane production decreased and even stopped on day 8. Then, it steadily increased after day 15 (Figure 6-3D) but never exceeded 5 NmL/g VS_{total}/d during the experimentation. During acidosis, a plateau is observed for cumulated methane yield (Figure 6-3A) reflecting the small daily methane production and the instability of anaerobic digestion. From day 6 to 10, the concentration of total VFA was the same (10 g/L) for S/I = 4 and 11 (Figure 6-3C) whereas a small difference in pH was observed (0.2 more for S/I = 4). Despite similarity for some parameters, one reactor recovered and the other failed. Indeed, between day 20 and 30, a decrease of VFA concentration occurred concomitantly to a pH increase and to a moderate methane production. pH for S/I = 4 was always higher than pH in S/I = 11 (Figure 6-3B). The difference of alkalinity between the two reactors on day 10 probably explains this phenomenon: 0.8 g $CaCO_3$/L more for S/I = 4 (Table 6-3). Alkalinity is related to the buffer capacity of the medium and must be sufficient. In stable reactor, alkalinity is frequently between 2 and 4 g $CaCO_3$/L (APHA, 1998). Alkalinity measurements in this study fitted with expected values (Table 6-3).

Table 6-3. Alkalinity and TVFA/alkalinity with TVFA in g HAc eq/L on day 1 and 10 of the Experiment I.

	Day 1		Day 10	
S/I *VS basis*	**Alkalinity (TA)** *g $CaCO_3$/L*	**TVFA/alkalinity**	**Alkalinity (TA)** *g $CaCO_3$/L*	**TVFA/ alkalinity**
11	4.24	0.36	3.28	2.46
4	3.87	0.33	4.12	1.95
2	4.67	0.22	4.57	0.59
1	4.40	0.27	4.83	0.40

6.3.2 TVFA/alkalinity as process stability indicator

Acid pH can inhibit methanogens or even kill them, leading to reactor failure. To better control the process, indicators are required to detect imbalanced reactions before a too detrimental pH drop. In this way, alkalinity is a crucial parameter. However, there are lots of different methodologies with different accuracy and suitability. Thus, a sufficiently accurate method must be used such as Ripley et *al.* (1986) technic, used in the current study (Lahav and Morgan, 2004). VFA, main responsible of pH drop must also be measured to detect imbalanced process. Several authors have suggested to study reactor stability using VFA but it appears that each reactor have its own "normal" levels of VFA (Ahring et *al.*, 1995). Notwithstanding, with liquid continuous stirred tank fed with Chinese cabbage silage and swine manure, reactors were stable with TVFA/alkalinity close to 1 but with VFA below 10 g/L (Kim and Kafle, 2010). Even if configuration of Experiment I was very different from this case, the limit of 10 g/L for VFA was also observed since above this limit reactor failed (S/I = 11) In the same way, with wheat straw and batch SSAD (22 % TS), reactor failure was also observed with VFA final level of 12.4 g/kg (Cui et *al.*, 2011).

Several studies highlighted the ratio between VFA and alkalinity as an efficient way to control an anaerobic digestion plant. It is considered that if TVFA (Total VFA in g HAc eq/L)/alkalinity (g $CaCO_3$/L) is between 0.3 and 0.4, anaerobic digestion process is stable (Lili et *al.*, 2011; Lossie and Pütz, 2008; Raposo et *al.*, 2006). This ratio is also referenced as FOS/TAC (derived from German language). Originally used in sewage sludge plant, this indicator has been successfully applied to control several liquid fermenters (Ehimen et *al.*, 2011; Raposo et *al.*, 2006). If some authors consider the limit of 0.6 as critical (Lossie and Pütz, 2008) others gave a limit at 0.8 (Callaghan et *al.*, 2002). Thereby, stable reactors were observed at 0.7 (Callaghan et *al.*, 2002; Kim and Kafle, 2010). However, several precautions must be taken to ensure a viable measurement (sample preparation, alkalinity measurement method…). Good initial and final TVFA/alkalinity ratio are not sufficient to indicate the absence of acidification period since this ratio must be observed in a dynamic way during anaerobic digestion; a high increase meaning a potential instability of the reaction (Kim and Kafle, 2010; E. Voß et *al.*, 2009) . The evaluation of the stability of the process must be completed by the methane yield because of the existence of inhibited steady state (Chen et *al.*, 2008; Kim and Kafle, 2010).

Without pretreatment, 49% of the BMP value can be expected after 26 days of wheat straw anaerobic digestion (Kusch et *al.*, 2011). Similar results were obtained after only 15 days for (without correction from methane inoculum production). S/I = 1 and 2 reached, respectively, 43% and 39% of the BMP value (247± 8 NmL/g VS). Thus, in the current study, inhibited steady-state is unlikely.

Most measured TVFA/alkalinity ratios in Experiment I (Table 6-3) were in the range of stable process (0.22 to 0.36 on day 1). On day 10, S/I = 2, with TVFA/alkalinity of 0.59 would be considered as instable with conventional limits. However, it was observed that anaerobic digestion was almost not affected.

Moreover, for S/I = 4 and 11, with TVFA/alkalinity near to 2 or higher, processes could be considered as highly instable with usual limits. It must be noticed that S/I = 4 was able to recover with a TVFA/alkalinity of 1.95 whereas S/I = 11 failed with TVFA/alkalinity of 2.46. In this experiment, a threshold of 2 for TVFA/alkalinity ratio was found to differentiate failure and SSAD able to recover.

Diverse studies with SSAD recorded very high TVFA/alkalinity ratio without instability or at least the possibility for the process to recover (Table 6-4). Thus, it seems that further studies are required to determine if usual limits fixed for TVFA/alkalinity ratio (in liquid process) are adapted to SSAD. pH and VFA measurements are generally carried out in leachate. However, SSAD is a very heterogeneous reaction therefore a neutral pH in leachate does not mean that no acidic areas exist in the substrate (Staley et *al.*, 2011). Martin (2001) hypothesed “acid-protected” areas for methanogens due to a biofilm structuration in SSAD process. This might be an explanation for higher observed ratios in SSAD. Staley et *al.* (2011) showed that if existing, such areas would be smaller than 3 cm diameter. Moreover, they pointed out the importance of acid-tolerant methanogens (i. e. *Methanosarcina bakeri*) to overcome acidification period. *Methanosarcina bakeri* is active at pH 5.7. In pure culture its optimum pH range is between 5 and 7. When

pH recovers the neutrality, metabolic activity is substantially enhanced, increasing methane production. Acid-tolerant methanogens certainly played a role in the recovery of the reactor with S/I=4. After their experiment (Table 6-4), Duan et *al.* (2012) considered without drastic pH drop, VFA accumulation would help the multiplication of methanogens.

Table 6-4. Stability limits for SSAD process with high TVFA/alkalinity ratios.

TVFA/alkalinity	Anaerobic digestion process	Substrate (composition in %TS)	Additional information	References
Final ratio at **1.6** or above for healthy reactors	Mesophilic **batch** at **20%TS** at S/I (VS basis) ratio of 4.1 or 6.2 for 30 d, no shaking	Fallen leaves (11.1% CEL, 11.5% Hemi, 22.7% LIG)	0, 2, 3.5 or 5% NaOH addition at the inoculation step	(Liew et *al.*, 2011)
Ratio followed during the whole process: Stable SSAD when **< 0.9** Acidification and recovery when **> 1.25 and < 5.4** Satisfactory methane production even with a peak between **3 and 4**	Mesophilic **batch** at **20%TS** at S/I of 2.2 (VS basis) for 48 d, slow shaking	Single or co-digestion of distiller's grain (22.9% Hemi, 19.9% Cel, 13.8% LIG) and food waste (12.3% Hemi, 4.4% Cel, 2.8% LIG) with several proportions tested	Initial alkalinity of 10 g/L $CaCO_3$ thanks to $NaHCO_3$ and $KHCO_3$ addition	(L. H. Wang et *al.*, 2012)
Initial ratio between **0.9 and 1.2** and**:** Final ratio **< 3** for reactors producing satisfactory methane Final ratio **> 3** for acidified reactor with no or very low methane production	Mesophilic **batch** at **20%TS** for 30 d at S/I (VS basis) of 1, 2 or 3	Mix of yard (24.3% Cel, 23% LIG, 9.7% Hemi) and food wastes (0, 10 or 20% VS) with several proportions tested		(Brown and Li, 2013)
Peak around **3**, average value of **2**, stable process (methane production and pH)	Thermophilic **continuous** process (stirred tank reactor, leachate recycle) at **20%TS** and 30% (w/w) inoculum for 60 d	Food Wastes from restaurant		(Forster-Carneiro et *al.*, 2008)
A decrease of organic loading rate allowed the recovery of stable process with satisfactory methane production after a peak between **0.8 and 1.1**	Mesophilic semi-**continuous** process (stirred tank reactor) at **15% or 20% TS**	Dewatered sewage sludge		(Duan et *al.*, 2012)
Possible recovery with a peak value of **1.95** Failure with a peak at **2.49**	Mesophilic **batch** with leachate recycle at **17-18%TS** S/I (VS basis) of 3, 4 or 11	Wheat straw inoculated with manure digestate		Current study
Peak value at **1.94** with a satisfactory methane production		Fungal colonized miscanthus and wheat straw		

For Liew et *al.* (2012), S/I =2 to 4 allowed similar methane production with wheat straw in 30 days batch SSAD (22% TS). With higher S/I a drastic diminution of methane production was observed. In mesophilic liquid batch reactors with wheat straw, high reduction of methane yield occurred also with S/I ratio greater than 4. In the current study (Experiment I), it was confirmed that S/I between 2 and 4 can be used for SSAD of wheat straw and that higher ratios seem inappropriate.

With wheat straw, some authors obtained low daily methane production (less than 1L/kg VS/day) during almost the 4 first days for S/I = 2 (Cui et *al.*, 2011; Liew et *al.*, 2012). In contrast, daily methane production was better in Experiment I with the same S/I ratio (Figure 6-3D). Leachate recycle can explain this result

because biological activities are limited by low moisture and nutrients. Leachate recycle favors also the mobility and repartition of degrading microorganisms (Kusch et *al.*, 2008). Methane production in Experiment I with S/I =2 reached 97 NL/kg at day 15, it was slightly better than the 90 L CH_4/kg VS (final production at day 30) obtained by Cui et *al.* (2011) or the 66 L CH_4/kg VS obtained by Liew et *al.* (2012) with 22% TS content. In Experiment I, correction of methane production by removing the endogenous production of inoculum was not done. However, differences with other studies seem too high to be only explained by this fact. More performant inoculum or more degradable substrate in Experiment I are a possible explanation for better production but it can also be due to leachate recycle and/or lower TS content used in Experiment I. The higher is the TS content, the lower is the methane conversion rate of the substrate and digestion duration are longer; as observed by Motte et *al.* (2013) with very high S/I (> 28) and small particles size (< 1.4 mm).

Even if leachate recycle can enhance acidification and must be reduced in the initial stage (Kusch et *al.*, 2008), Li et *al.* (2011) considered that some areas receive less acidic leachate and thus can regenerate the process. For S/I = 4, a lower recycling rate in initial stage would allow to limit acidification. In the same way, sequential batch reactors that recycle leachate between new and mature reactors allow more stable reactions. VFA produced during start-up of a new reactor are re-circulated via leachate to an older reactor for methanogenesis. Leachate of older reactor, acid free and with enough alkalinity is recirculated to the new reactor.

6.4 Experiment II: fungal pretreatment for SSAD

As reactor with S/I = 2 seemed to start easily (in contrast to the one with S/I = 4), as S/I = 4 had recovered and since productivity per reactor is improved with substrate amount; it was chosen to use a S/I ratio of 3 for Experiment II. In this experiment a fungal pretreated straw was used (reactor FWS). This one was inoculated with fungal colonized miscanthus pellets, thus a control reactor with raw straw and fungal colonized miscanthus (reactor cMWS) was carried out. Finally, a reactor with miscanthus pellets and wheat straw constituted another control (reactor MWS).

6.4.1 Substrates anaerobic digestibility

Few studies reported methane production improvement after fungal pretreatment of lignocellulosic substrate (Chapter 1, Table 1-4) but they are generally led sterilely at lab-scale whereas in the current study, fungal pretreatment was realized at pilot-scale with 400 g of wheat straw. Wheat straw used in Experiment II and Experiment I was the same. The greater TS content in Experiment II was due to freeze-drying (Table 6-1). Fungal growth on wheat straw was successful, with lot of white areas corresponding to mycelium of *P. brumalis* BRFM 985. However, fungal growth was not homogeneous (pictures in Annex 12) and a longer pretreatment time seemed to be required in order to pretreat all the straw. Large untreated areas can explain why TS and VS were similar for pretreated and untreated straws (Table 6-1). BMP of pretreated straw was a

bit lower than for untreated straw (25 NmL/g VS less, Table 6-1) whereas up to 40% methane yield improvement can be expected with *Polyporus brumalis* BRFM 985 pretreatment (Chapter 1). Culture conditions, in this study, were not enough optimized to increase straw BMP. Culture conditions cannot be compared to those obtained in the optimization step (Chapter 4) since they are lot of differences between the two processes (as further discussed later, Chapter 7). BMP increase can be obtained when lignin is sufficiently attacked and when carbohydrates losses are small. Pretreatment conditions are as important as an efficient fungal strain to improve substrate digestibility (Wan and Li, 2012). Despite the BMP decreased, if fungal pretreatment enhances sufficiently the methane production rate for a given anaerobic digestion duration, methane production would be increased. Since hydrolysis is the limiting step for lignocellulosic substrates when anaerobic digestion is stable (Bertrand et *al.*, 2006), improvement of methane production rate must be due to hydrolysis enhancement after fungal pretreatment (lignin removal, etc.). Methane production rate was investigated in Experiment II for FWS compared to controls. Controls contained miscanthus pellets. BMP of colonized miscanthus was also lower than uncolonized miscanthus (12 NmL/g VS less, as further discussed) probably due to carbohydrates consumption to ensure fungal growth. In this case, the goal was the obtaining of healthy and consequent fungal mycelium able to colonized wheat straw, an improvement of the miscanthus BMP was not expected. There was a small decrease in VS for colonized miscanthus that explained the slightly lower VS content of the corresponding batch reactor (6 g, Table 6-2).

6.4.2 SSAD start-up phase

During start-up phase all reactors experienced acidic pH (<6.5) but the harshness of acidification varied among reactors (Figure 6-4B). The most affected batch was cMWS with the most acidic pH (near 5.5) and the longer period under low pH (6 days). Batch which encountered the least difficulties was FWS, with higher pH and the shortest period under acidic conditions. Low pH directly affected daily methane production (Figure 6-4C). Each time pH felt under 6.5 (day 1 for cMWS, day 2 for MWS and day 4 for FWS), a stop or a decrease in daily methane production occurred (Figure 6-4C). VFA production peaks were observed around day 5 for all reactors (Figure 6-4A). After this day, daily methane production and pH increased thanks to the consumption of VFA. cMWS alkalinity was measured on day 5 at the VFA peak, leading to a TVFA/alkalinity ratio of 1.94 (< 2, critical value in Experiment I). A rapid recovery was observed since the critical value differentiating recovery from failure was not reached. Total VFA concentrations in leachate were lower than 10 g/L that appeared as a possible other critical value in Experiment I.

During the first two days, daily methane production was maximal for cMWS, followed by MWS and then by FWS. This production is very likely due to compounds with no (or very small) need of hydrolysis to be converted into methane. Those compounds were more present in cMWS because fungi hydrolyzed lignocellulosic polymers. They were the less numerous on FWS, probably because fungi had more time to

use them for fungal metabolism: 15 days growth on straw and 25 days on miscanthus for fungi of FWS against 0 day on straw and 10 days (growth before straw inoculation) on miscanthus for fungi of cMWS.

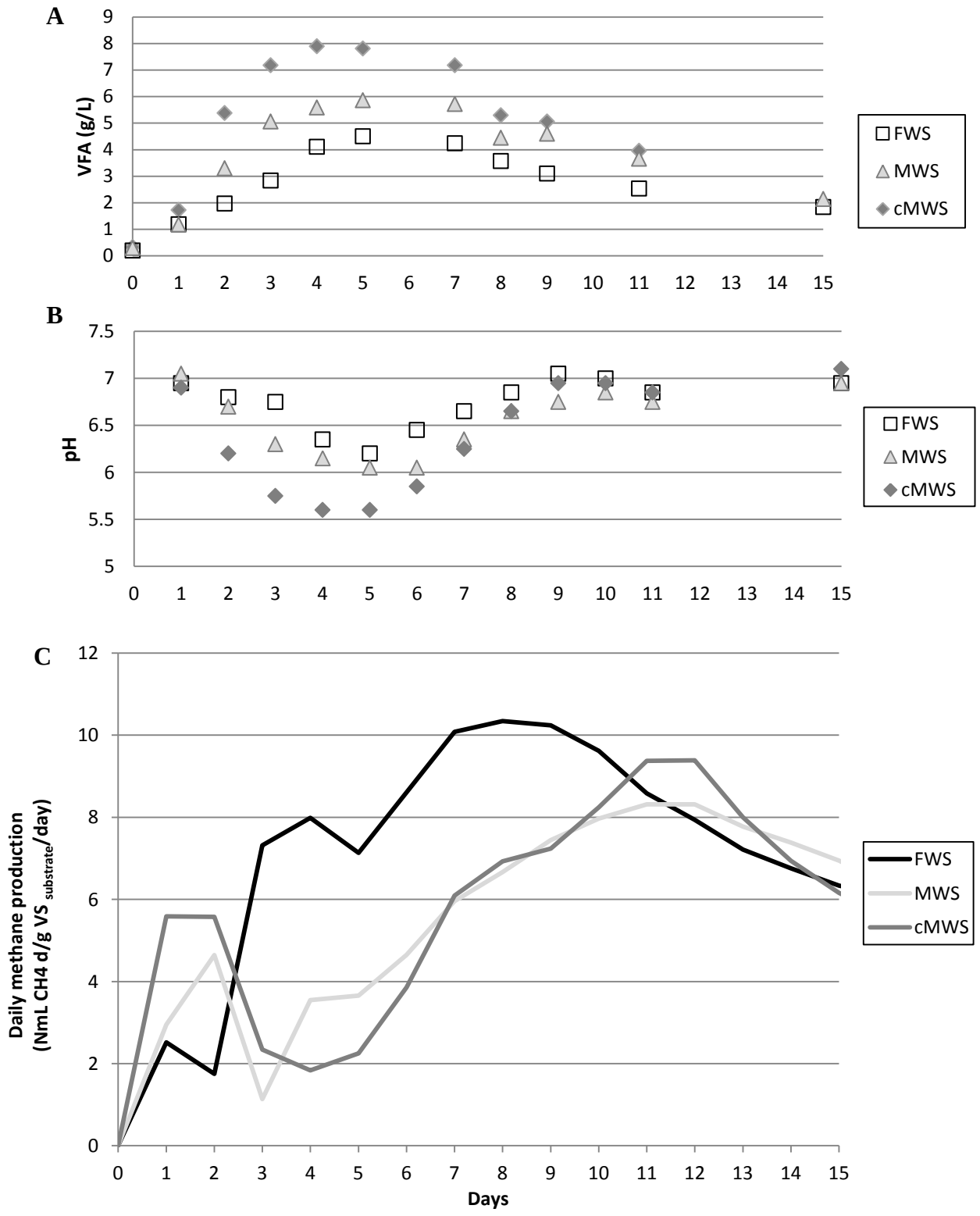


Figure 6-4. Parameters of anaerobic digestion during the start-up phase for Experiment II in function of time.

A. Leachate global VFA concentration. B. Leachate pH. C. Daily methane production per g of VS.

Maximum daily methane production was a bit highest for FWS than for other reactors. Moreover, production peak occurred on day 8 for FWS whereas the peak was on day 12 for cMWS and MWS (Figure 6-5A). Thus, methane production rate was enhanced for FWS. However, no conclusion can be drawn about the influence of fungal pretreatment on hydrolysis rate during anaerobic digestion since it was not the rate limiting-step. Differences between reactors are principally due to acidification period (methanogenesis limiting) for cMWS and MWS. Methane content of the biogas during steady production stage was similar between reactors and reached 55-60% that is in accordance with reported values in literature for SSAD of wheat straw (Cui et *al.*, 2011). Methane yield reported to pretreated VS was also better for FWS (Figure 6-5). Therefore, FWS is more biodegradable than controls (MWS and cMWS). The increase biodegradability and the effect of fungal pretreatment had led to a slight decrease in final total carbon for FWS compared to controls (Table 6-5).

Table 6-5. Methane production and final nitrogen amount for Experiment II.
[a, b, c] samples followed by the same letter are not significantly different (ANOVA with $\alpha = 0.1$).

		Solid inoculum	Miscanthus + Straw (**MWS**)	Colonized Miscanthus + Straw (**cMWS**)	Fungal pretreated straw (**FWS**)
Expected yield calculated with BMP values (CH_4/g VS)			237	236	222
Methane production at day 60	(CH_4/g VS)		181	181	204
	(% of final expected value)		76.4	76.7	91.9
Yield at day 127, experimentation stopping	(CH_4/g VS)		230	215	254
	(CH_4/g TS initial)		219	203	203
Final TKN for solid digestate (%TS)		2.37	2.40	2.44	2.36
Final NH_4^+ in leachate (mg/L)		9.53	5.52	9.20	18.26
Final TC for solid digestate (%TS)		37.61[a]	44.93[b]	44.88[b]	42.97[c]

6.4.3 Methane production of fungal pretreated straw in SSAD

Despite acidification period, overall methane productions were not affected (Figure 6-5): at day 127 (experiment stopping), they are very close to expected values (based on BMP reported in Table 6-1). However, during fungal pretreatment mass losses occurred (≈20% TS for FWS in this study) and must be considered to evaluate the global efficiency of the process. This mass loss can be taken into account expressing methane production per g of initial TS (before pretreatment TS): at day 127, methane yield (NmL/g TS initial) is similar between reactors cMWS and FWS (Figure 6-5).

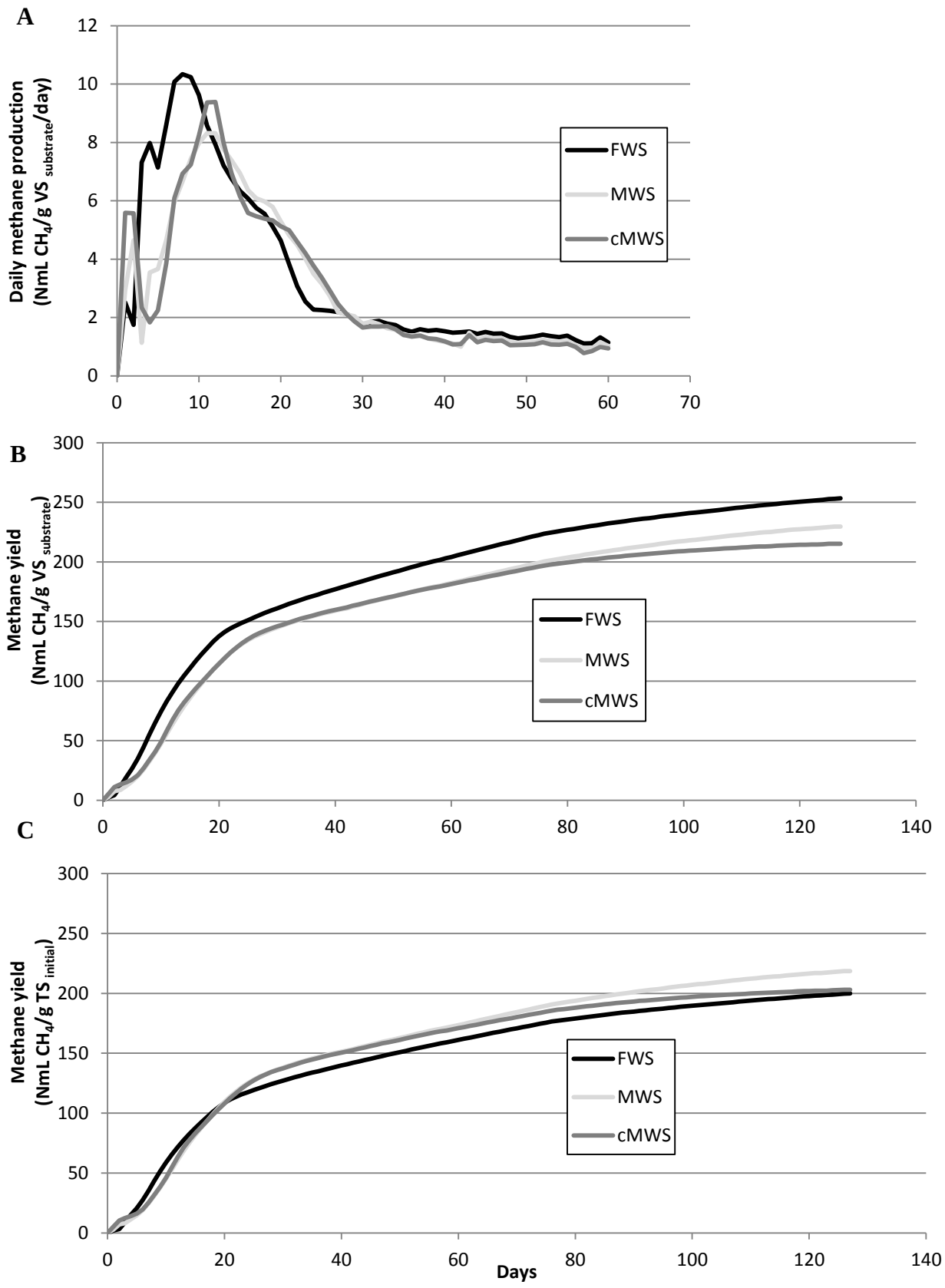


Figure 6-5. Methane productions for Experiment II.
A. Daily methane production reported to substrates VS. B. Methane production per g of substrate VS. C. Methane production reported to TS before fungal inoculation.

Reaction duration of 60 days seems adapted to SSAD of wheat straw in batch. If substrate amount is not limited, considering a reaction duration of 60 days and the same amount of straw (pretreated or not), 12.6% more methane (/ g VS) can be obtained with pretreated straw compared to untreated substrate (Table 6-5). In this configuration, fungal pretreatment may allow an additional income. Even with suboptimal pretreatment conditions, results of the current study are encouraging. With methane yield per g of initial TS, a bit less methane (10 NmL/g TS initial) is obtained for FWS than for controls in 60 days (Figure 6-5C). During the first twenty days, methane production per g of initial TS is nevertheless better for FWS owing to a better start-up phase. An efficient start-up phase corresponds to a more rapid CH_4 production that will reduce quickly concentrations of undesirable compounds such as explosive oxygen. In this way, the possibility to valorize methane by injection into grid arrives earlier. Moreover, start-up phase can last until 250 days (with certain couple process/substrate) (Karthikeyan and Visvanathan, 2013). Thus, the reduction of this period can really have an economical attractiveness. Pretreatment would be useful for reactor with short reaction duration (< 20 d) or to favor good reactor start-up.

If wheat straw amount is limited and with a given size of reactor, longer reaction duration can be applied for pretreated straw owing to mass losses during pretreatment. Considering same conditions as in Experiment II, with a reactor size of 4.7 t wet weight (inoculum + substrate), and 6 t TS wheat straw that can be pretreated or not, annual methane production is similar per VS amount whether substrate is pretreated or not (Table 6-6). However, total methane production per year (i. e. not reported to substrate amount) would be lower of around 1300 Nm^3 for FWS because pretreatment conditions were not optimal in Experiment II. If pretreated straw was compared to untreated one, higher methane production loss would have been reported since BMP of miscanthus (colonized or not) is lower than the one of wheat straw (Table 6-1). Optimization of pretreatment conditions is primordial, notably to limit mass losses.

Table 6-6. Estimation of annual methane production in the case of limited straw amount.

	Untreated	Fungal treated
Wheat straw (t TS)	6	4.86
Solid inoculum (t wet weight)	21.9	17.8
Batchs/year	6.1	4.8
Average reaction duration (d)	60	75
Annual methane production (m^3)	6302*	4998

*based on methane production at day 60 for MWS and cMWS

In addition to the better start-up phase and the biodegradability improvement, another interest of fungal pretreatment for lignocellulosic biomass is the diminishing of C/N by the consumption of carbon and the conservation of nitrogen during pre-treatment. This reaction combined with N input by fungal inoculum leads to an increase TKN content for colonised substrate (Table 6-1), as also observed by Bisaria et *al.* (1983) and Zeng et *al.*(2011). C/N optimal ratio for anaerobic digestion is between 20 and 35 and is dependent of the feedstock. Higher ratio can lead to nitrogen limitations, increased VFA accumulation and decrease of process stability and biogas yield (Li et *al.*, 2011; Sialve et *al.*, 2009). Wheat straw generally

requires co-substrate to decrease its C/N ratio as its C/N can reach value as high as 161 (McKendry, 2002b). If co-substrates rich in nitrogen are not available on site, N supplementation can constitute an additional cost that would be reduced if fungal pretreatment is carried out. At the end of the SSAD, solid digestates have a similar NTK content but NH_4^+ concentrations in leachate reflect the N content of initial substrate: FWS > cMWS > MWS (Table 6-5). Leachates coming from SSAD of fungal pretreated straw have potentially a better fertilizing value since available nitrogen is a primary need for plants growth.

6.5 Conclusion

Firstly, this study confirmed that S/I equal to 2-3 are required for wheat straw SSAD. Moreover, SSAD processes that experienced a TVFA/alkalinity lower than two and VFA concentrations lower than 10 g/L were able to recover from acidification. Studies with other substrates and inocula would allow to determinate if such limits can be generalized to SSAD batch with leachate recycle.

Secondly, fungal pretreatment wheat straw led to small methane production losses, after taking into account substrate mass losses during the pretreatment. However, pretreatment conditions were suboptimal. Thus, fungal pretreatment may be still interesting for biogas plants; notably if its cost is controlled (possibility to produce fungal inoculum on site…). Research efforts must continue to propose an optimized fungal pretreatment at pilot-scale: cheap, with low mass losses (especially of carbohydrates) and high delignification.

Chapter 7.

Results overview and general discussion

Objective

- To compare results obtained in the different chapters results between them and with other information generated by partner projects

Table of content

7.1 An efficient pretreatment

Among pretreatments used to improve digestibility of lignocellulose, biological pretreatments are largely under investigated and often represent few lines in dedicated review (e. g. Carvalheiro et *al.* 2008; Talebnia et *al.* 2010; Monlau et *al.* 2013). WRF appear as the most promising among biological pretreatments. If their advantages are easily recognized (low energy consumption, safety of use, environmental friendly), their long reaction time puts a brake on their development as pretreatment. However, lignocellulose is frequently stored and it can be taken advantages of this period. The second inconvenient of WRF is the consumption of cellulose and hemicelluloses whereas these polymers are converted to biogas during anaerobic digestion. For this reason, Zheng et *al.* (2009) even considered biological pretreatment as less attractive commercially. However, the lack of data concerning costs of these pretreatments does not allow concluding about their feasibility.

In the current work **best result after WRF pretreatment** corresponds to 44% more methane (/g VS pretreated) than control straw (uninoculated straw in glass columns). This improvement was obtained for *P. brumalis* pretreated straw during 21 days in glass columns at 28°C (Chapter 1). It **is similar to** the **improvement reported** on wheat straw **with other pretreatments** that could be easily applied to full-scale (Chapter 1): 53% enhancement for grinding (0.4 mm), 43% and 67% increase with NaOH (10% at 40°C for 24 h and 10% at 100°C for 30 min, respectively) (C. Sambusiti et *al.*, 2013; Sharma et *al.*, 1988). Moreover, as less inputs are required (energy, water and chemicals), WRF may be more interesting economically. Finally, they are environmental friendly (no sodium addition which may be detrimental for digestate valorization…). WRF and grinding pretreatments can be realised on solid substrates without or with limited addition of water. However, grinding can enhance reactors acidification in SSAD (Motte et *al.*, 2014b). Chemical pretreatments generally require important addition of water amount even if investigations about dry chemical pretreatment are beginning (Abdellatif Barakat et *al.*, 2014a). In those conditions, WRF pretreatments fit particularly to SSAD that allows higher substrate loading.

However, **taking into account mass losses** the efficiency of *P. brumalis* BRFM 985 best pretreatment felt to 21% more methane per gram of initial TS (Chapter 1). This amount is still quite interesting, especially if input requirement are low (Berglund and Börjesson, 2006). Mass losses are rarely reported in the few studies dealing with WRF pretreated lignocellulose for anaerobic digestion (Chapter 1) even if they are necessary to evaluate pretreatment performances. To our knowledge, only one study was realized on wheat straw with fungal pretreatment for biogas production. Among 22 basidiomycetes, *Pleurotus florida* was chosen to pretreat sterilized wheat straw for 90 days with 75% moisture content at 25°C. After batch anaerobic digestion, 27.9% more methane was obtained (v/w pretreated TS). The overall pretreatment efficiency was negative since 42.5% of mass losses occurred during pretreatment (Müller and Trösch, 1986). Results of the current study are far better. Furthermore, mass losses is not only a concern for WRF pretreatments since organic matter losses between 1 and 13% have been reported for diverse silage technics (Herrmann et *al.*, 2011). Losses during the storage are frequent but generally not quantified. With grass, mass losses (water and organic matter) were estimated at 14% during wilting in the field and storage (Blokhina et *al.*, 2011). As

water losses are also included, this study cannot be directly compared to the 20% TS losses often observed with WRF pretreatments (Chapter 1, and observed in Chapter 6).

Among the 63 screened strains by Zhou et *al.* (2015b), *P. brumalis* was one of the most interesting strains to improve sugars yield 72 h enzymatic hydrolysis. **In literature**, *P. brumalis* was little investigated compared to other WRF such as *Phanerochaete chrysosporium* or *Pleurotus ostreatus*, especially if mycelial growth on substrate is considered rather than application of enzymes extracts. Some studies reported the pretreatment of lignocellulosic biomass with *P. brumalis* to improve enzymatic hydrolysis of cellulose (saccharification, first step for ethanol production). However, those studies compared several WRF and considered ***P. brumalis*** as **less efficient than other tested strains** (Hatakka, 1983 on wheat straw; Lee et *al.*, 2007 on Pinus densiflora). It is possible that *P. brumalis* BRFM 985 is more effective than other tested *P. brumalis* which originated from other strains banks. Another possibility is inadequate culture conditions for *P. brumalis* since they really influence pretreatment (Chapter 1, 3, 4 and 6). In this sense, the screening led for STOCKACTIF project was realized with all strains in the same conditions. Thus, strains considered as effective were efficient in these screening conditions. With other culture conditions and with the same strains, it is strongly possible that other strains would have been considered as interesting to pretreat lignocellulose. When the goal is to identify the best strains, **screening with several culture conditions must be carried out** even if tested strains would be less numerous. This methodology was notably followed by Zadražil (1985) who studied *in vitro* dry matter digestibility for ruminants feed treated with hundreds strains grown at 22, 25 and 30°C during 30 and 60 days. Due to lack of time, it was not possible, in the project, to test different culture conditions on the large number of strains. The priority was put on the exploration of the diversity of the CIRM-CF bank ("Centre International de Ressources Microbiennes-Champignons Filamenteux"). For this reason some Brown-Rot Fungi (BRF) were included in the screening although BRF are known to little modify lignin and to attack carbohydrates. Consequently, BRF are globally less effective than WRF for lignocellulose pretreatment even if one screened BRF strains was efficient (*Antrodia malicola* BRFM 1200) (Zhou et *al.*, 2015b). In addition, retained **inoculation** technique for screening was **using mycelia disc** that consists to cut agar plug on the periphery of a petri dish where fungus had actively grown. This technique **might not be suitable for strains comparative studies** because of the different growth rates. Mycelia density on the agar plug is dependent of fungal strain and thus inoculum amount is not standardized. Mycelia suspension or Cellophane Film Culture (CFC) technique (agar plate overlaid with cellophane film to ease the separation) should be preferred as they allow the quantification of fungal biomass in the inoculum (Yoon et *al.*, 2014).

Despite these observations, *P. brumalis* **BRFM 985 has a lot of advantages** since this strain was not only efficient to pretreat wheat straw (for anaerobic digestion applications) but also miscanthus (for ethanol production applications) (Zhou et *al.*, 2015b). This efficiency on both lignocellulosic biomasses let assume the possibility to pretreat other substrates as results would not be too dependent of the couple substrate/fungus used. The screening was carried out in deep-well with addition of starter solution before fungal inoculation (200 mg glucose/g dry straw and 18.4 mg of tartrate diammonium/g TS). In order to

measure the BMP of pretreated substrates, interesting strains were cultivated once again in deep-well. Some of them received four times less starter amount than for the screening, notably because this addition can decrease the efficiency of the pretreatment and represent a supplementary cost for industrial scale. In these conditions, certain strains were unable to grow. In contrast, *P. brumalis* BRFM 985 can be cultivated without starter addition that constitutes a competitive advantage for this strain. *P. brumalis* BRFM 985 seems ubiquitous as it can also be reflected by the large range of usable growth temperature (at least 8-35°C) or the capacity to grow with low moisture content (1.9 WW/TS i, Chapter 4). Its rapid growth is also interesting (personal communication from S. Zhou). Finally, it is probably not a coincidence if *P. brumalis* will be the first fungus to be cultivated in space (NASA, 2015; Valley Christian Schools, 2014).

It is very likely that the **maximum methane improvement** for *P. brumalis* BRFM 985 treated straw **was not reached**. In the current work (Table 7-1), best measured improvement (44% CH_4 more/g VS pretreated and 21% CH_4 more/g TS initial) are expressed by comparison to control straw (uninoculated straw in glass columns: NIC). Slight starter amounts were added for these measurements (25 mg of glucose/g TS and 2.5 mg of tartrate diammonium/g TS). As remaining starter after pretreatment can be converted into methane with anaerobic digestion, the comparison to control straw whether than untreated straw (UWS) is relevant. Moreover, UWS was not available for this series of BMP-tests. From an industrial point of view, results must be compared to UWS when possible. During the optimization of pretreatment with *P. brumalis* BRFM 985, best measured improvement compared to control straw (NIC) were smaller (20% CH_4 more/g VS pretreated and only 5% CH_4 more/g TS initial) as shown in Chapter 4 and Table 7-1. The comparison with biological control (NIC) allows quantifying fungal action. Pretreatment process (glass column) even without fungal inoculation increases straw BMP (Chapter 4). To evaluate all aspects of the **pretreatment** (physico-chemical and fungal) a **comparison to untreated straw** is possible since no starter were added: 43% CH_4 more/g VS pretreated were obtained compared to UWS instead of 20% CH_4 more compared to NIC. Taking into account mass losses, best BMP obtained during optimization step (206 NmL/g TSi for sample 9, Table 4-3) corresponded to 13% more methane compared to UWS. In this case, the pretreatment can be considered as efficient but it would not be mainly due to fungal inoculation.

Table 7-1.Variation of methane production from *P. brumalis* BRFM 985 pretreated wheat straws compared to control according pretreatment conditions.

	Selection step: Chapter 1		Optimization step: Chapter 4	SSAD reactors: Chapter 6	
Pretreatment system	Deep-well	Glass column		Pilot-reactor	
Added glucose amount with starter solution (mg/g straw)	200	25	0	0	
Anaerobic digestion system	BMP tests			BMP tests	Batch reactors SSAD
Control considered	Improvement TS_{init} or VS_{pretr} (for starter amount correction)	NIC		UWS	MWS
CH_4 reported to pretreated VS	- 16%	+ 44%	+ 20%	- 10%	+ 10%
CH_4 Reported to initial TS	- 30%	+21%	+ 5%	- 29%	- 8%

Finally, a negative **impact of starter addition** regarding pretreatment efficiency was observed in Chapter 1. However, in Chapter 1, effects of glucose addition were investigated for pretreatment in deep-well during 14 days with 0 or 50 mg glucose/g straw whereas best methane improvement was observed for pretreatment in

glass columns with culture duration of 21 days and 25 mg glucose/g straw (and slight nitrogen addition). In literature, it was already explained (Chapter 1) that there is a controverse about the impact of glucose on pretreatment since both positive and negative effects were observed. With moderate addition of glucose in pulp mill wastewaters containing lignin and slight nitrogen amount, Wu et *al.* (2005) observed an increased secretion of ligninolytic enzymes by *P. oestreatus* and a faster delignification whereas high glucose amounts decreased ligninolytic enzymes and β-glucosidase activities and thus the COD removal from liquid medium. Consequently, lower results obtained for the optimization step compared to selection step might be due to the different starter amount between those steps (no starter addition for optimization). To check this point, a repetition of the best pretreatment (21 days in glass columns with slight starter amount) and the same pretreatment without starter must be carried out followed by anaerobic digestion.

In the experiment design, the maximum calculated BMP for treated straw (with metals addition) was obtained for longest fungal **culture duration** (≈ 20 days). Optimum methane production was not reached in the experimental domain whereas maximum dry matter losses would have been obtained (Annex 4). It can also be noticed that 21 days culture duration were required to obtain the maximum methane improvement of the current work (Chapter 1). For these reasons, culture duration longer than 20 days may lead to consequent methane improvement (per g of initial TS). To obtain the optimum value for BMP, especially regarding the pretreatment duration, few more experiments can be carried out since the chosen experimental design allows the addition of new experimental data points. However, for anaerobic digestion step, anaerobic inoculum would be slightly different of the one used in Chapter 4 and it is not sure that supplemental measurements would allow a good extrapolation of data. Since maximum calculated BMP in the experimental domain does not correspond to highest tested temperature or moisture content, it would not be necessary to increase the experimental domain for those variables.

During the optimization of pretreatment, other culture conditions could have been tested such as aeration. In literature review (Chapter 1), it was discussed that oxygenation led to a more rapid delignification for *P. brumalis* but selective delignification was not necessarily improved. However, more culture conditions tested would require numerous experimental points that are difficult to manage with limited time and materials. Moreover, such a work would lead to a better characterization only at lab-scale (culture in glass columns with 20 g straw). **With another SSF system, optimum culture conditions** to improve BMP of pretreated straw would **be different** than in glass columns. Pretreated straw was obtained in a pilot-reactor (Table 7-1) but the pretreatment was not optimized and was inefficient: large uncolonized areas and diminishing of the straw BMP after pretreatment (Chapter 6).

Finally, to fully access the efficiency of fungal pretreatment for anaerobic digestion, it is also important to have **an efficient SSAD**. Anaerobic digestion in reactor is necessary to investigate pretreatment efficiencyfor full-scale plants (Chapter 6). It can notably allow studying anaerobic digestion rate since its improvement would be another interest of WRF pretreatment. It can be interesting from an industrial point of view, especially because some lignocellulosic residues require a long time to be transformed by anaerobic

digestion. Müller & Trösch (1986) observed the maximum daily methane production for untreated samples two days after the one for treated samples (90 d pretreatment of wheat straw with *Pleurotus florida*). An increment of the initial hydrolysis rate for fungal treated poplar was measured by Yang et *al.* (2013). It can lead to a better methane production rate as hydrolysis is often the limiting step of anaerobic digestion (Yang et *al.*, 2007). The acceleration of the hydrolysis can be caused by the enhanced surface exposure of fungal treated material. To have an economic interest, improvement of methane production rate must occur for anaerobic digestion duration shorter than solid-retention time of full-scale plants. Otherwise, no improvement of methane production is obtained. In the current study (Chapter 6), methane production rate was improved for anaerobic digestion duration lower than twenty days but it was due to acidification in controls rather than hydrolysis enhancement (increased porosity…) by fungal pretreatment. **Another experiment** with better conditions in all reactors (methanogenesis not limiting) **is required** in order **to study the possible enhancement of hydrolysis step of anaerobic digestion with fungal pretreatment**. Such an improvement can lead to higher methane production rate. Moreover, around 200 g of wheat straw efficiently fungal pretreated, i. e. with an improved BMP compared to UWS, are necessary for further experiment (as it was not the case in Chapter 6).

To carry out an efficient SSAD, the main parameter to determine is S/I in batch systems but diverse strategies can also be tested, e. g. optimum leachate recycle rate (Chapter 6), thermophilic temperatures or even studies in continuous reactors. Co-digestion strategies must especially be explored as an improved methane yield can be obtained due to synergisms (better C/N, nutrients supply…) (Mata-Alvarez et *al.*, 2000). It is very **likely that C/N for fungal pretreated straw is still too elevated** for an optimum anaerobic digestion. The lowest measured C/N with straws issued from selection step was 68 with straw pretreated in glass columns with BRFM 1048 (Annex 13) whereas optimum C/N for anaerobic digestion is between 20 and 35. In SSAD reactors (Chapter 6), the amount of substrate TS corresponded to more than 2/3 of the whole TS content (substrate + inoculum). Considering these proportions and a C/N equals to 68 for pretreated straw, a C/N inferior or equal to 35 cannot be reached whatever the C/N of the inoculum ($68 \times \frac{2}{3} + \frac{1}{3}x \leq 35$).

Evaluation of pretreatment economic feasibility is dependent on increase output (methane yield) and input (additional cost due to pretreatment). Lab-scale data fail to **evaluate full-scale costs** (Carlsson et *al.*, 2012) and pretreatment feasibility cannot be fully assessed without certain details about the context of the process application (Chapter 6). The industrial trial of the STOCKACTIF project will certainly provide useful data to better characterize a full-scale process, especially from a technico-economic point of view. The anaerobic digestion of the industrial trial is scheduled between January and March 2016.

7.2 WRF pretreatment scale-up: the contaminations challenge

With **thirteen days** of pretreatment at pilot-scale (200 g straw * 2), large areas without fungal colonization were reported on pretreated straw (Chapter 6, pictures in Annex 12) whereas colonization in glass columns

was more homogeneous (pictures in Annex 14). This phenomenon can illustrate the necessity to find optimal culture conditions for each culture system. The principal difference between the two processes is that one was sterile (glass columns) whereas the other was not (pilot-scale). Straw particles size, another parameter influencing SSF, was different between pilot-scale (few centimeters) and lab-scale (4 mm). This experiment (August 2014) was carried out before the obtaining of optimization step results (November 2014) and parameters (WW/TS = 4.7 and temperature = 30°C) were chosen a priori. Metals addition was used but without $MnSO_4$ (not available at this time). **The major problem** encountered during the project to cultivate *P. brumalis* on hundreds grams of wheat straw **was** a **fungal contamination** (picture without magnification in Annex 15). The contaminant fungus is white (as *P. brumalis*) at the beginning of its growth then hundreds of black spores are scattered into the substrate and the mycelium takes a grayish aspect (Figure 7-1). Those phenotypic characteristics suggest an *Aspergillus* contamination, which will be confirmed (or not) by sequencing (still on-going). Thus, it was not possible to study influence of culture conditions in another SSF system such as the pilot-reactor.

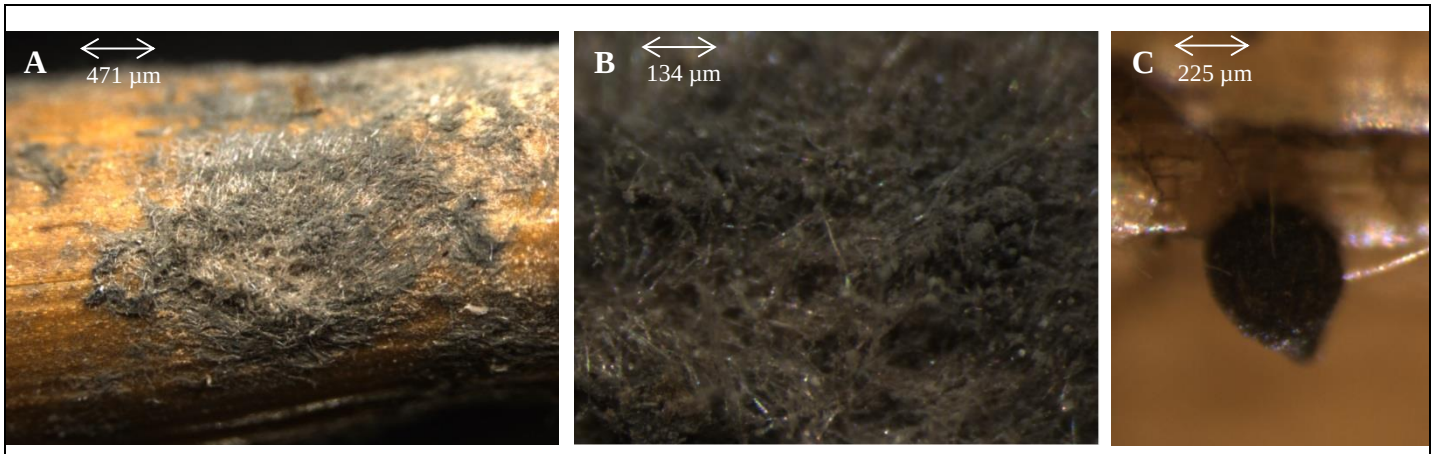


Figure 7-1. Stereomicroscopic observations of the fungal contaminant in the pilot-scale pretreatment.
A. Grayish aspect of the contaminant on wheat straw (magnification x 27.4). B. Numerous black spores scattered by the contaminant (magnification x 101). C. Spore of the fungal contaminant (magnification x 101).

Contaminations occurred on autoclaved straw cultivated under clean but not sterile conditions (as described Chapter 26, 6.2.1). More than ten experimentations failed in two years. A pretreatment at pilot-scale takes approximately one month (17 days for inoculum cultivation and 15 days of wheat straw pretreatment). Once, almost no contamination occurred (Chapter 6, pictures in Annex 12), this pretreated straw was used for SSAD in reactors. It is interesting to note that contaminations were observed whatever the origin of wheat straw (different harvested years or fields). As **return of experience on the LBE pilot concerning contaminations**, several observations must be highlighted. It can be difficult to maintain moisture content during the pretreatment but it is essential to succeed. Moreover, free water may favor growth and spore germination of the contaminant (Gil-Serna et *al.*, 2014). Pretreatment reactor design must be though to avoid areas difficult to clean that can host contaminants between two experimentations. In the same way, large pipes must be preferred to small ones and reactor parts must be easy to change. A particular attention must be provided to inputs (water, air), and to strategies to ensure their cleanliness (bleach in water, air filtering

with sterile filters…). Effective means of disinfection must be implemented in case of contamination but also before each pretreatment. At the LBE, kärcher cleaning and sometimes fumigation with a fungicide were used (Fumispore® OPP). Kärcher cleaning allows a mechanical action to take ofF impurities and the heating action (120°C almost 20 min) is potentially effective to kill contaminants.

A certain trend for *P. brumalis* BRFM 985 to grow on peripheral straw and with small height of straw pile evokes a high oxygen need. However, increase oxygen intake is not sufficient to succeed since multi-criteria are implied in fungal growth and pretreatment. High amount of fungal inoculum helps to have a rapid fungal development limiting contaminations. To facilitate inoculation, INRA of Marseille (BBF) advocated inoculum cultivation on miscanthus pellets. Low contamination level was observed with very high inoculum amount (one unit for two units wheat straw). At industrial scale, more inoculum would mean more miscanthus pellets, higher cost and more difficulties to handle it. Thus, adequate inoculum amount must be found. Finally, it was also tried once to use starter (glucose + N) for cultivation at pilot-scale of *P. brumalis* BRFM 985. Starter addition has probably favored contaminations as numerous ones were observed after this pretreatment. In test tubes, it was checked that the contaminant was not present in straw with a culture under sterile conditions on autoclaved straw. It was also noticed with autoclaved contaminated straw that the contaminant can grow and sporulate after autoclaving. Sometimes, the cleanliness of the pretreatment system was checked before inoculation using petri dishes in the reactor and swabbing (wall, water…).

In literature, it is advised to decontaminate the substrate with a short anaerobic fermentation step before inoculation. Dilute aromatic fungicide or moderate acidic pH during culture would be helpful to favor WRF which are able to digest aromatic structures and to acidify the substrate (Chapter 1, 1.3.2). Those strategies were not tried but dilute fungicide seems not environmental friendly and acid pH would be more adapted to limit microbial contaminations than fungal ones. Even if the presence of the contaminant in wheat straw was not turned out; a particular attention must be paid to wheat straw decontamination, especially because the contaminants can grow after autoclaving step. Trials of straw thyndallization could have been carried out. This technic is especially adapted to fight against microbial spores and consists in several cycles of moderate heating (e. g. 30 min at 60°C) each 24 hours. The principle is based on spore germination followed by their elimination when they are in a less resistant form. This technic is probably also efficient against fungal contaminations as successful subsequent fungal culture were reported (Meier et *al.*, 2011; Rehbein and Koch, 2011). For industrial scale trials, Soufflet partner will try soon to cultivate *P. brumalis* BRFM 985 on ionized, pasteurized and raw wheat straw under sterile conditions. In most studies, substrates are sterilized but to a larger scale, it can be costly and not very easy to handle (notably because of pressure). In contrast, Akin et *al.* (1995) succeeded to obtain similar biodegradability improvement for grass digested with ruminal microorganisms and pretreated with three different fungi whether it was on sterile or unsterilized Bermuda grass stems. With unsterilized yard trimmings treated with *Cyathus stercoreus*, Zhao et *al.* (2014a) obtained the same methane production as with sterilized material. In their study, unsterilized substrate was inoculated with sterile pre-colonized material (one unit for 4 or 9 units of dry matter unsterile substrate). In the current study, inoculation was done with sterile material with one unit for four units dry matter sterile substrate, in

the general case. The degree of disinfection required is fungi dependent and substrate dependent (nature and amount of endogenous flora). It may be useful to keep microorganisms that do not compete with WRF development but disturb colonization by harmful microorganisms (Ficior et *al.*, 2006). However, it seems not easy to carry out without carbohydrates consumption.

For further investigations on fungal pretreatment, the tolerance of WRF to contamination must be considered in strain screening or selection steps (e. g. with a culture on pasteurized straw whether than autoclaved…). The capacity to grow quickly is also important since once the substrate is colonized, contaminations have some difficulties to implement. Finally, one pretreatment trial was carried out in the LBE's pilot with miscanthus straw. The growth was successful as large areas with dense white mycelium (*P. brumalis* BRFM 985) were observed without black spores from contaminant (picture in Annex 16). In the same way, other project partners did not have difficulties for *P. brumalis* BRFM 985 culture on miscanthus. Thus, miscanthus appears as a more adapted substrate for this fungal strain. Methane improvement is possible since in literature, 5% more methane (NmL/g TSi) were obtained with miscanthus pretreated with *C. subvermispora* (Vasco-correa and Li, 2015). Differences between wheat and miscanthus straws (chemical composition…) exist but crops technical itineraries are also different. Contrary to wheat straw, miscanthus does not spend several days on soil that can potentially contaminate it. However, it is an energy crop whose use is regulated in France, especially for anaerobic digestion application, due to ethical problems raised by energy crops (Art. L. 541-39., 2015).

Wheat straw contaminations led to a decrease in BMP (Figure 7-2), probably due to carbohydrates consumption. Whatever the contamination levels BMP (NmL/g VS pretreated) was similar and slightly lower than the one of raw straw (Figure 7-2). Taking into account mass losses, differences between raw and contaminated straws would be higher. Mass loss was only measured for straw without contamination and estimated at 20% (Chapter 6); they may be higher for contaminated straw. Pretreatment conditions were inadequate since non contaminated treated straw had a slightly lower BMP compared to raw straw (UWS). Only fungal contamination was visible (to the naked eye) at pilot scale but a consumption of carbohydrates by bacteria is also a possibility to explain similar BMP whatever fungal contamination level at pilot-scale. Finally, the heterogeneity of the substrate (Chapter 6) would also explain those results. To avoid this problem, straw was ground to 1 mm before BMP measurements but sample portions taken before grinding can have been not sufficiently colonized by fungi. The impact of contamination in SSAD reactor may be different since BMP tests are not sufficient to predict full-scale performance (Carlsson et *al.* 2012 and Chapter 6).

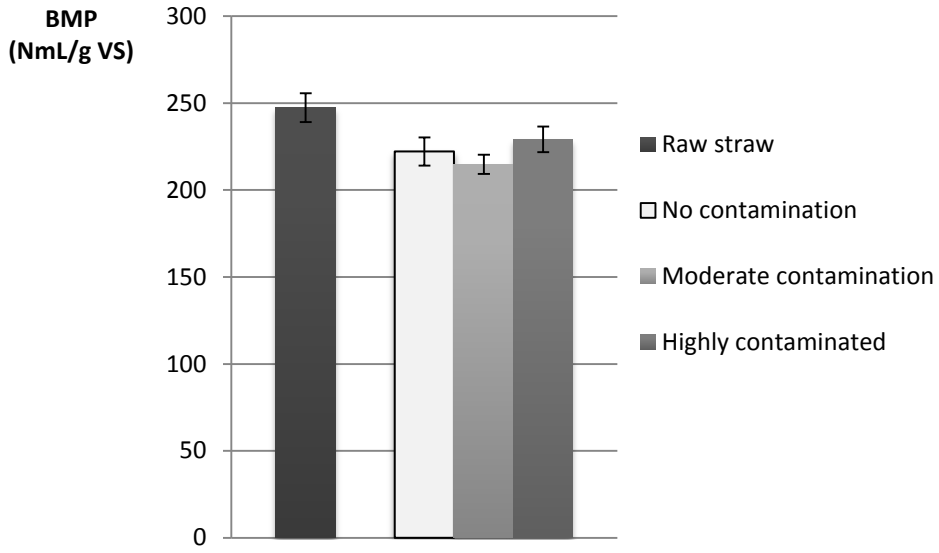


Figure 7-2. BMP of straws (1mm) pretreated in the LBE pilot-reactor with different level of contaminations and compared to BMP of raw straw (1mm).

7.3 WRF pretreatment for ethanol and methane production

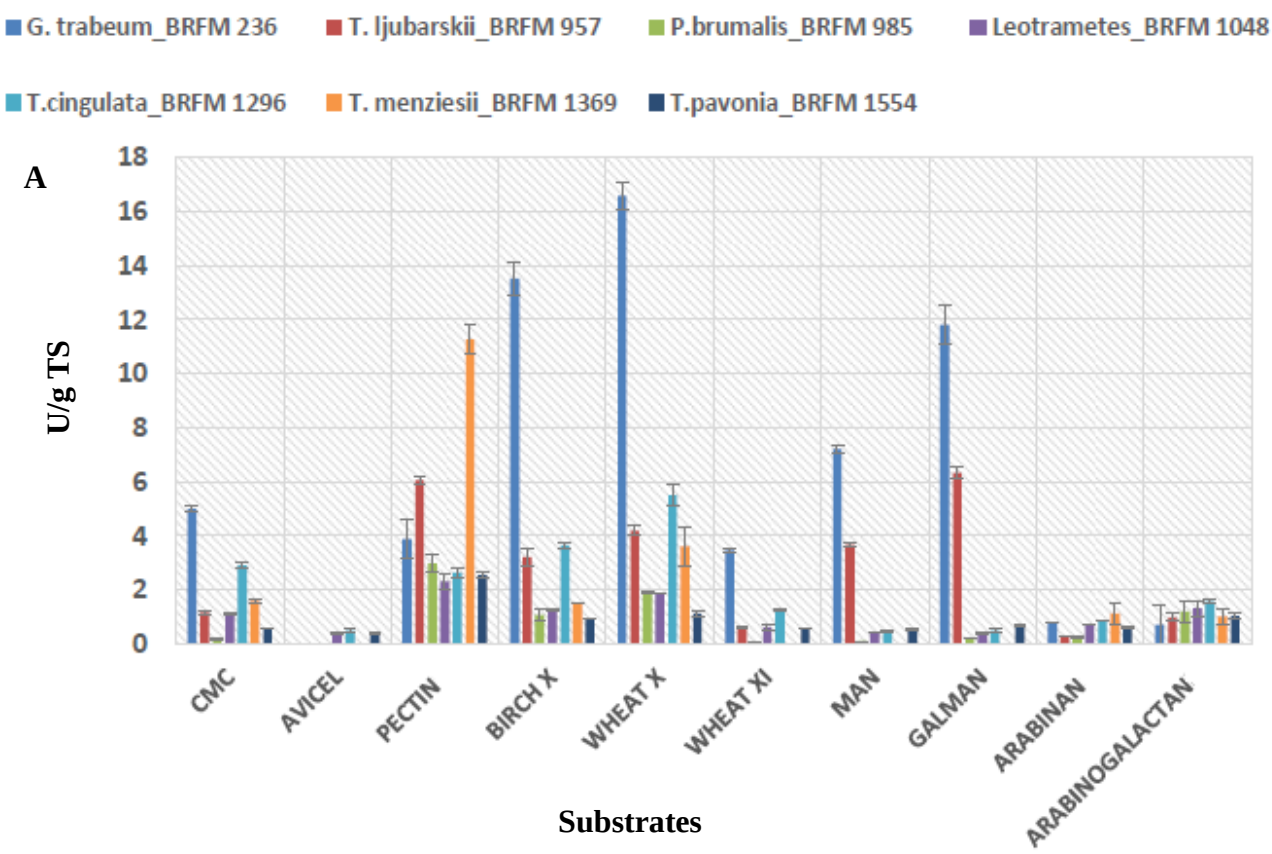
In STOCKACTIF project, **S. Zhou studied the interest of the WRF pretreatment for ethanol production**. For this purpose, delignification is required to maximize glucose yield after hydrolysis. Ethanol is presently produced from glucose issued from cellulose, even if efficient xylose fermenting strains are emerging (Kuyper et *al.*, 2005). To recognize the fitness of WRF for ethanol production, Lignin Decomposing Selectivity (LDS) ratio can be used. It is defined as the ratio of lignin degradation over cellulose loss (Tian et *al.*, 2012).

There were diverse **concordant observations between the present work and the one of S. Zhou** (BBF laboratory). A patent was deposited in France by BBF and LBE laboratories about fungal pretreatment of lignocellulosic biomass for bioenergy production (Gimbert et *al.*, 2014). It is based on results obtained in glass columns with five different WRF strains for a 3-weeks pretreatment duration (samples from selection steps, without *T. cingulata* BRFM 1296 excluded for intellectual property reason). On these pretreated wheat straws, S. Zhou studied fungal biomass, mass losses, straws composition and enzymatic hydrolysis (digestibility) of cellulose. Fungal enzymatic activities at the end of the pretreatment were also measured. They concerned both the hydrolytic system (glucosides hydrolases on xylan, Avicel, pectines…) and the ligninolytic one (Laccases, MnP…). Finally, BMP of pretreated straws, measured at the LBE (Chapter 1), are also included in the patent (Gimbert et *al.* 2014; Zhou et *al.* 2016, *under writing*).

Table 7-2. Composition changes of pretreated wheat straws compared to control.
Loss values are calculated taking into account the initial content of each component in WS.
N. A.: Not Available; * Selectivity LDS: Lignin losses (%)/Cellulose losses (%) (Zhou et *al.*, 2016, in preparation)

Fungal Strains	Mass losses (%)	Fungal biomass (mg/g)	Component loss (%)			Selectivity LDS*	Cellulose digestibility (%)
			Cellulose	Hemicelluloses	Lignin		
T. ljubarskii BRFM 957	33 ± 3	73 ± 6	40 ± 1	44 ± 1	51 ± 1	1,3	40.8 ± 1.2
P.brumalis BRFM 985	19 ± 1	50 ± 3	16 ± 2	28 ± 2	40 ± 1	2,5	54.3 ± 2.0
Leiotrametes sp. BRFM 1048	20 ± 2	48 ± 9	18 ± 2	22 ± 2	35 ± 2	2	36.3 ± 1.6
T. menziesii BRFM 1369	25 ± 2	64 ± 4	28 ± 2	30± 2	37 ± 1	1,3	36.5 ± 1.7
T.pavonia BRFM 1554	21.5 ± 0.9	20 ± 2	25 ± 1	25 ± 2	34 ± 1	1,4	29.8 ± 0.9
G. trabeum BRFM 236	11.2	N. A.	11	19	8	0.7	N. A.
Control NIC	3.5 ± 0.7	-	-	-	-	-	32.6 ± 1.4

For all tested strains, S. Zhou found an efficient delignification that increased cellulose digestibility (except for *T. pavonia* BRFM 1554) compared to control (Table 7-2). Digestibility of cellulose is the percentage of glucose released after enzymatic hydrolysis reported to whole glucose contained in tested straw. *P. brumalis* BRFM 985 and *Leiotrametes sp.* BRFM 1048 were the most selective strains regarding LDS, attacking lignin and preserving cellulose (Table 7-2). With limited mass losses, ***P. brumalis* BRFM 985 appeared as the most interesting strain to pretreat wheat straw for ethanol production** since remaining cellulose is highly digestible (more than 20% increase compared to NIC) (Table 7-2). Enzymatic activities vary a lot during fungal culture (Gutierrez et *al.*, 1995). However, at the end of the pretreatment, *P. brumalis* BRFM 985 produced higher ligninolytic enzymes along with lower glucosides hydrolases activities as compared to other fungi (Figure 7-3). Moreover, whereas tested strains consumed similar cellulose and hemicelluloses amounts, *P. brumalis* BRFM 985 had a slight preference for hemicelluloses consumption in those culture conditions (Table 7-2). *T. ljubarskii* BRFM 957 degraded extensively all polymers from lignocellulose leading to higher mass losses than for other strains (33% against 20% in average). Culture duration for *T. ljubarskii* BRFM 957 was probably too long. In accordance with the significant degradation of holocelluloses, glucosides hydrolases activities at the end of the pretreatement were the highest for this fungus (Figure 7-3). Concerning the Brown-Rot *G. trabeum* BRFM 236, as expected it attacks less the matter (11% mass losses) with a preferential consumption of carbohydrates marked by a low LDS (Table 7-2), high glucose hydrolases and low ligninolytic activities at the end of the pretreatment (Figure 7-3).



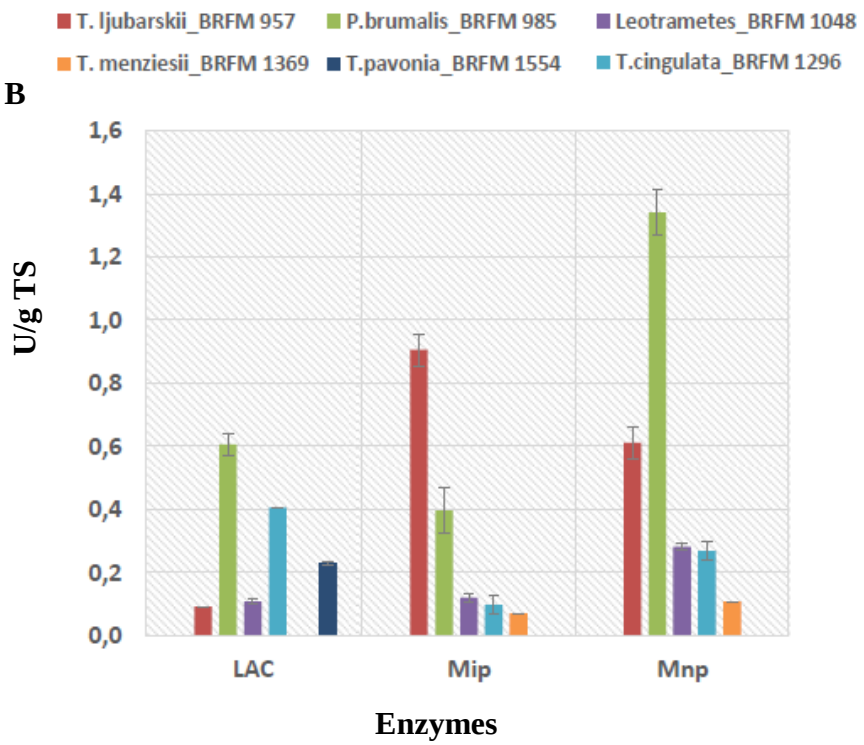


Figure 7-3. Enzymatic assays after 21 days of pretreatment for samples from the selection step.
A. Glucosides Hydrolases activities on various substrates. *CMC: Carboxymethyl cellulose; X: Xylan; XI: Insoluble Xylan; Man: Mannan, GalMan: GalactoMannan*. B. Ligninolytic enzymes activities: *LAC, laccases; Mip, Manganese independent peroxidase; Mnp, Manganese peroxidase*.(Gimbert et *al.*, 2014).

Cellulose digestibility was related to BMP (R^2 = 0.7542, n=6, Figure 7-4A) even if BMP is also issued of hemicelluloses degradation that is not quantify with enzymatic cellulose hydrolysis.

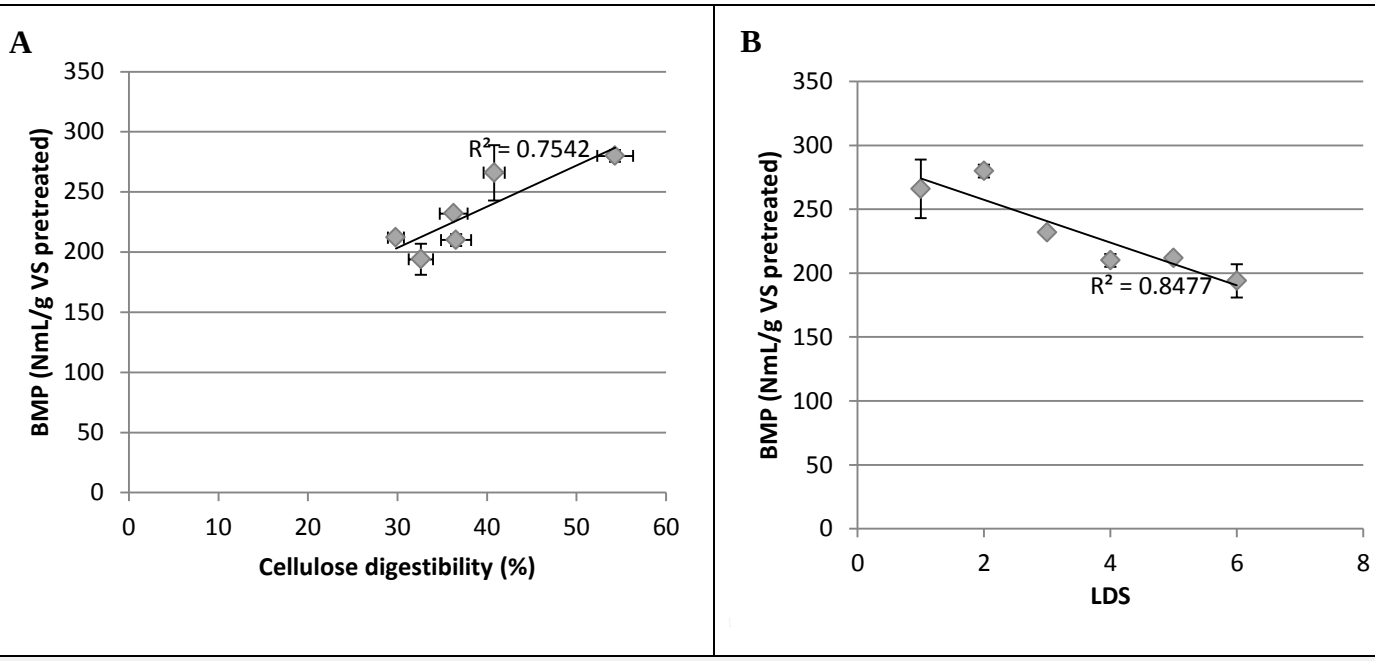


Figure 7-4. Correlations between BMP and substrate characterizations for non inoculated wheat straw and WRF pretreated straws from Table 7-2 (selection step).
A. BMP versus cellulose digestibility. B. BMP versus Lignin Decomposing Selectivity (LDS).

Cellulose digestibilities were similar to the one of NIC for most samples except for *T. ljubarskii* BRFM 957 and *P. brumalis* BRFM 985 that were higher (Figure 7-4A and Table 7-2). In accordance, with those results BMP (NmL/g VS pretreated) for pretreated straws were not very different from the one of NIC (Figure 7-4A, Table 3-2), except for *T. ljubarskii* BRFM 957 and *P. brumalis* BRFM 985.

As already evoked, *T. ljubarskii* BRFM 957 led to an extensive degradation. The remaining straw had consequently a high BMP per g of pretreated VS reflecting the accessibility of the substrate but after taking into account high mass losses (33%), impact of pretreatment on BMP is negative (lower than for control straw). Taking into account mass losses thanks to the BMP expressed per gram of initial TS, *P. brumalis* BRFM 985 was also found to be the best strain (21% more methane than NIC). With LDS and mass losses, **S. Zhou observed that the second most selective strain was *Leiotrametes sp*. BRFM 1048**. This strain led to a poor improvement of cellulose digestibility comparable to the one obtained with the inefficient *T. menziesii* BRFM 1369 but hemicelluloses losses were highest for *T. menziesii* BRFM 1369. **The second best BMP (NmL/TS i)** was obtained for pretreated straw with *Leiotrametes sp*. BRFM 1048 (Table 3-2, Chapter 1). However, this BMP was not significantly different from the one of control straw.

The correspondence between BMP and LDS (R^2=0.8477, n=5, Figure 7-4B) is not surprising even if BMP are also function of hemicelluloses (not considered with LDS) since a correlation seems to exist between cellulose and hemicelluloses losses (R^2 = 0.76, Figure 7-5A). The LDS ratio is reminiscent of PS/LIG ratio used to evaluate impact of fungal pretreatment with pyrolysis and that was correlated to BMP for straws

from the selection step (Chapter 5). Without *P. brumalis* BRFM 985 pretreated samples, the correlation between cellulose and hemicelluloses losses and its slope is improved (R^2 = 0.96, Figure 7-5A) that can reflect the slight preference for hemicelluloses consumption compared to cellulose in those culture conditions. The low consumption of cellulose is also illustrated (Figure 7-5B) by the sharp decline of the existing correlation between cellulose and lignin losses and of its slope when BRFM 985 is included in data (R^2 = 0.56 against R^2 = 0.97 without BRFM 985). **Lignin selective degradation is linked to a certain cellulose conservation** in the tested conditions since the behavior of BRFM 985 is not different from other strains concerning the concomitant attack of hemicelluloses with lignin (Figure 7-5C).

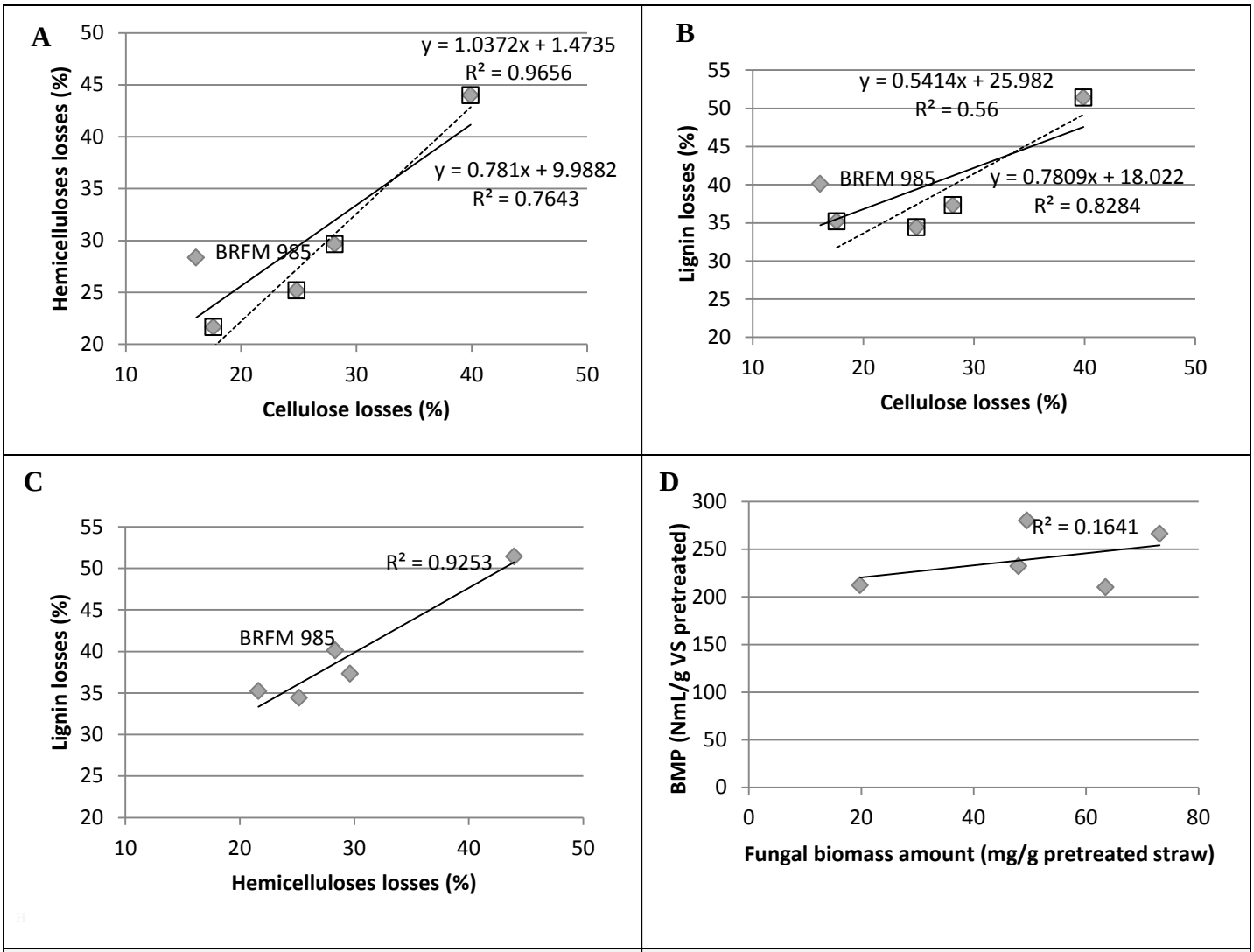


Figure 7-5. Characterizations of pretreated straws used for the patent deposit.
A. Correlation between hemicelluloses and cellulose losses compared to untreated straw. B. Correlation between lignin and cellulose losses compared to untreated straw. D. Absence of correlation between BMP and fungal amount for straw pretreated with different strains.

Moreover, it was shown that the amount of fungal biomass on wheat straw contribute slightly to BMP improvement (Chapter 4). However, BMP is also function of substrate modifications that are more important for long culture duration (Chapter 4) and dependent of fungal strain (Chapter 1). Furthermore, different fungal biomasses had different BMP reflecting their different composition. Consequently, BMP (NmL/g VS pretreated) is not correlated to fungal biomass amount (R^2=0.16, Figure 7-5D) for straws treated 21 d with different strains.

7.4 Further relationships between BMP and substrate characteristics

7.4.1 Samples from the optimization step

Concerning the optimization of *P. brumalis* BRFM 985 pretreatment in glass columns, the link between BBF's characterization work was already exposed since it can explain the degradability of pretreated straw in anaerobic digestion (Chapter 4). To go further, the existing correlation (R^2 = 0.82) between hemicelluloses and cellulose losses for *P. brumalis* BRFM 985 pretreated straw in several conditions was plotted (Figure 7-6A). The slight preference for hemicelluloses consumption observed in the selection step (Figure 7-5A) and in deep-well with different starter amounts (Chapter 1) is no more observable in those conditions since cellulose losses are generally higher than hemicelluloses losses (Figure 7-6A). Correlations between carbohydrates losses and lignin losses (Figure 7-6B) reveal a **non-selective lignin attack during the optimization step** and explain low BMP values for pretreated straws.

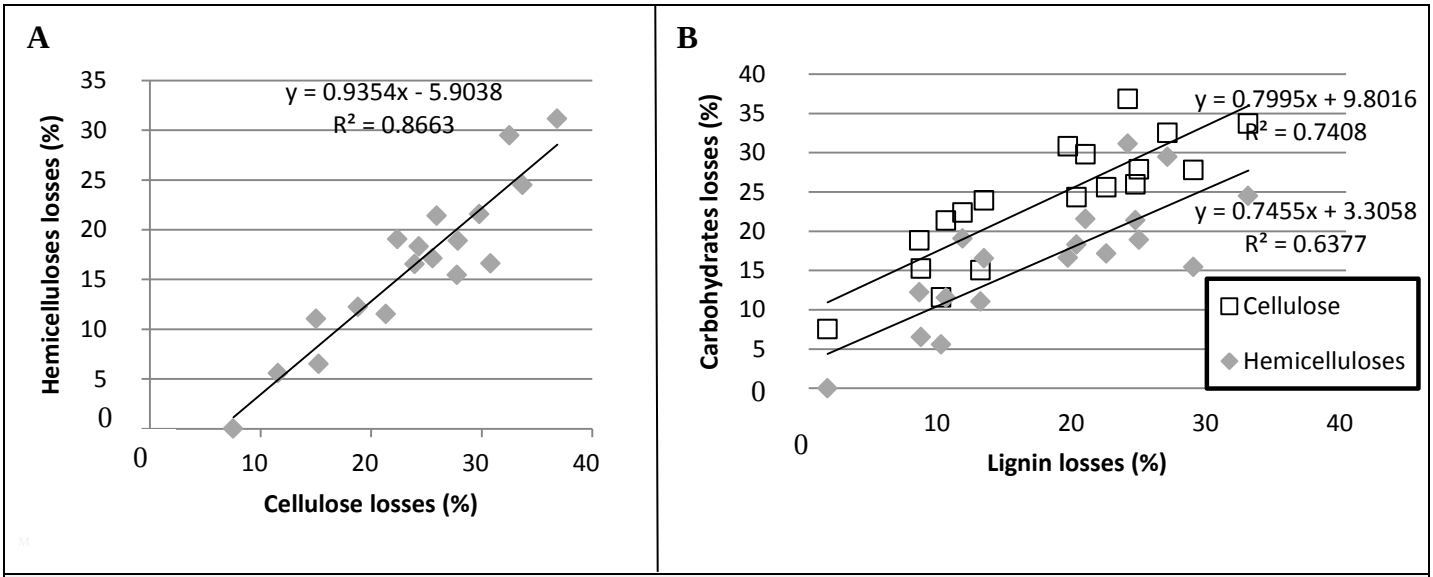


Figure 7-6. Correlations between cellulose, hemicelluloses and lignin losses for samples from the optimization step.
A. Hemicelluloses losses versus cellulose losses. B. Lignin losses versus carbohydrates losses.

One of the main differences between **selection step** and optimization step was the use of a slight starter amount for selection steps (25 mg glucose/g straw). In Chapter 1, few samples were cultivated in deep-well with different glucose amounts (0; 50; 200 and 400 mg glucose/g straw). It was concluded that starter addition would have a negative impact on the pretreatment, especially by limiting the delignification, in a strain dependent way. For BRFM 985 in deep-well, lignin amount in pretreated substrates was not significantly different without glucose or with an addition of 50 mg glucose/g straw (Figure 3-3, Chapter 1). In glass columns with slight glucose amount (25 mg/g), S. Zhou found insignificant cellulases activities after pretreatment with *P. brumalis* BRFM 985 (activities on CMC and Avicel in Figure 7-3A). It is **possibly due to an inhibition of cellulases activities caused by glucose** (as it was suggested for *P. brumalis* BRFM 985 behavior in deep-well, Chapter 1). BRFM 985's cellulases were probably more active in the optimization step (not measured) since cellulose consumption was emphasized. This increase of cellulases activities might be due to the absence of starter in the optimization step. However, lot of other culture parameters

(culture duration…) could have played a role on cellulose consumption. Lignocellulosic composition and cellulases activities for straw pretreated without and with slight starter amount are required to conclude.

Correlations between hemicellulloses losses and celluloses losses do not exist for all WRF as observed by Salvachúa et *al.* (2013) with *Irpex lacteus*. This fungus seems to consume preferentially cellulose concomitantly with lignin rather than hemicelluloses (absence of correlation between hemicelluloses and lignin losses). Carbohydrates losses seem unavoidable during pretreatment. It would be interesting to determine among lignin selective strains which co-substrate to lignin consumption is preferable among cellulose or hemicelluloses for BMP improvement of the pretreated substrate. In the current study, strains that do not consume hemicelluloses concomitantly to lignin were not observed that is why no conclusion can be drawn about this point.

The **relationship between cellulose digestibility and BMP** (NmL/g VS pretreated) was investigated using optimization step data since a **correlation** between the two parameters was found for straws from the selection step (Figure 7-4A, $R^2 = 0.7542$). The correlation exists also for samples from the optimization step ($R^2 = 0.7233$, n=18, Figure 7-7A). Cellulose digestibility determined in 72 hours would be an efficient way to explore quickly anaerobic degradability of fungal pretreated substrate. In this study, six months were required to reach BMP values. Cellulose digestibility is notably dependent of lignin (Figure 7-7B) and cellulose crystallinity (not measured for optimization step) that both influence BMP (Monlau et *al.*, 2012b). Holocelluloses digestibility would be of even greater interest to explore pretreatment efficiency for anaerobic digestion.

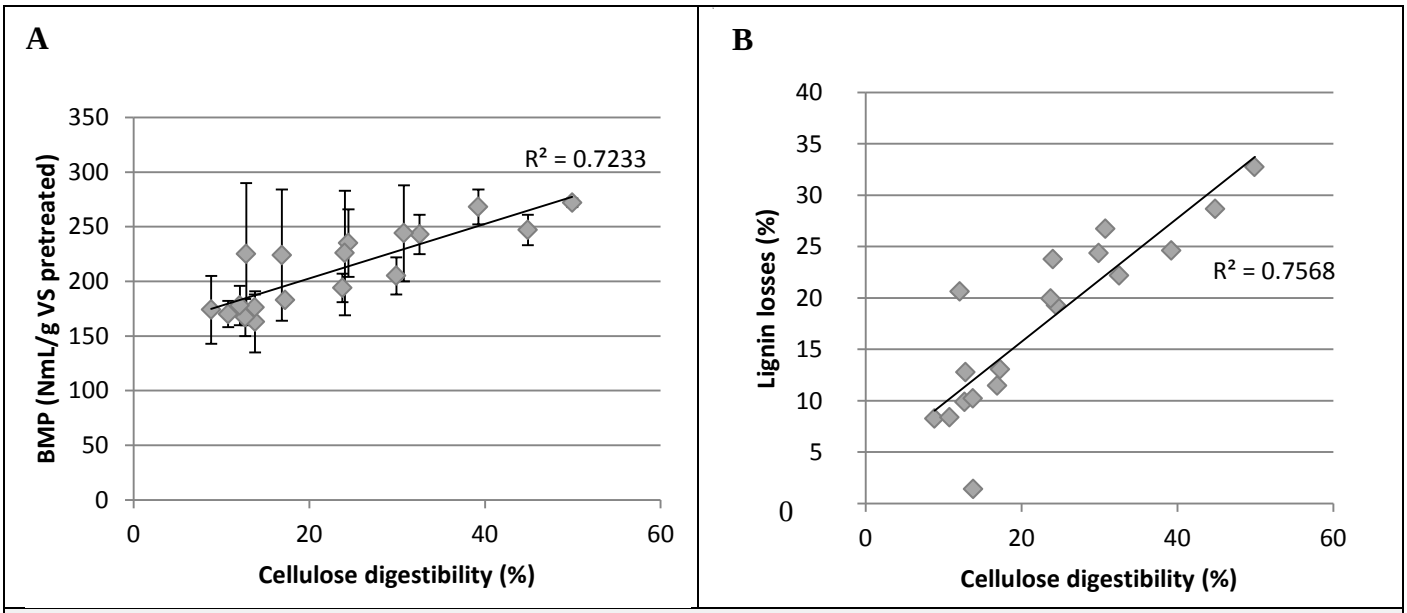


Figure 7-7. Correlations with cellulose digestibility for samples pretreated in the optimization step.
A. Cellulose digestibility versus BMP. B. Cellulose digestibility versus lignin losses (%).

In Chapter 5, a correlation between PS/LIG determined with pyrolysis (PS/LIG-pyr) or NREL method (acid hydrolysis) was found with BMP (NmL/g VS) of pretreated straws from selection step and few samples from the optimization step. In the optimization step, **a poor correlation** was observed between PS/LIG determined with NREL **(PS/LIG NREL) and BMP values** ($R^2 = 0.42$, n=18, Figure 7-8). The separation of

samples pretreated with or without metals addition only slightly improved correlation for samples with metals (R^2 = 0.5, n=10, Figure 7-8) and an absence of correlation was observed for samples pretreated without metals. Such a distinction seemed relevant since metals addition significantly increase lignin losses and consequently the BMP values. PS/LIG ratio would perhaps be more adapted to compare pretreated samples in the same conditions but with different strains (as it was mostly the case for Chapter 5).

However, for pyrolysis study (Chapter 5), no correlation was observed between PS/LIG-pyr and PS/LIG NREL. Thus, a correlation between PS/LIG-pyr and BMP values for samples from the optimization step would be still possible. Unfortunately, only few samples from the optimization step were available for py-GC-MS measurements (as explained later). With py-GC-MS studies, a particular attention must be paid to improve the measurement repeatability for heterogeneous samples (milling, replicates, etc.).

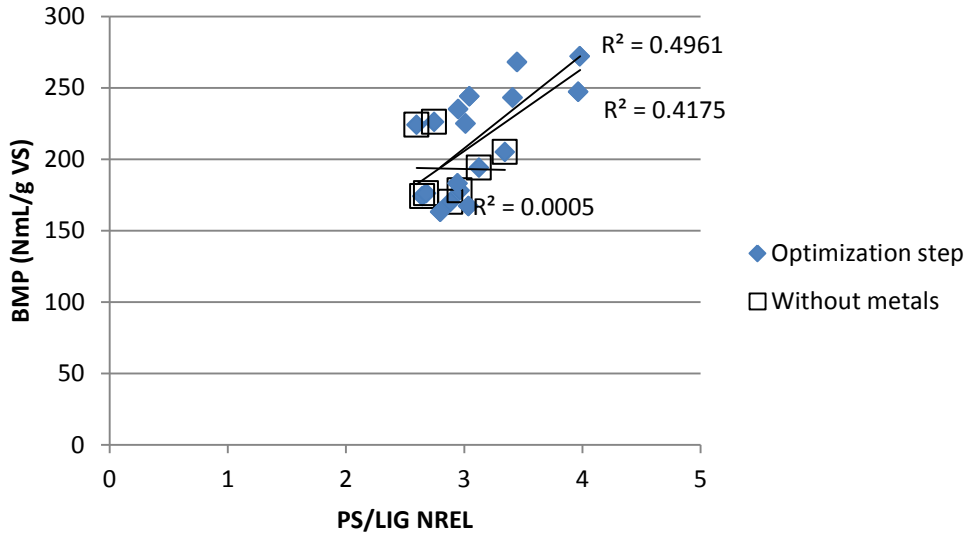


Figure 7-8. Relationship between BMP and PS/LIG NREL for samples pretreated in the optimization step.

7.4.2 Samples from the selection step

Characterizations are essential to understand pretreatment mechanisms in order to evaluate the efficiency of the reaction and finally to try to control it. Various characteristics are known to influence BMP of lignocellulosic biomass (Chapter 1). Among them, lignin amount and S/G were determined for samples from the selection step. Moreover, TKN content, also measured, can be linked to protein content (Li et *al.*, 2001) that is known to influence BMP values. Finally, cellulose crystallinity is equally an important parameter impacting BMP values. It was assessed using the widespread Fourier-Transform InfraRed (FTIR) spectroscopy. In addition, to evaluate compositional changes between samples, the relative areas of carbohydrate peaks at 1375 and 1158 cm^{-1} against lignin peak at 1512 cm^{-1} (aromatic C=C) were calculated to obtain ratios called PS1/ LIG and PS2/ LIG, respectively. In carbohydrate peaks there is no significant contribution from lignin (Yang et *al.*, 2009), they correspond to hemicelluloses and cellulose (C-H group at

1375 cm^{-1} and C-O-C at 1158 cm^{-1}). Crystallinity determination with FTIR is based on the measurement of Lateral Order Index (LOI) that corresponds to areas ratio of peak at 1430 cm^{-1} (CH_2 of crystalline cellulose) on peak at 898 cm^{-1} (amorphous cellulose) (Spiridon, 2011).

Impact of fungal pretreatment on substrate parameters that are susceptible to modify its BMP was studied on straws pretreated with several strains in glass columns (selection step). Some characterizations were carried out and used to build a Principal Component Analysis (PCA): BMP; PS/LIG and S/G determined with py-GC-MS; PS1/LIG, PS2/LIG and LOI determined with FTIR; Total Carbon (TC) and Total Kjeldahl Nitrogen (TKN) both expressed in percentage of pretreated TS. Several data from S. Zhou (BBF laboratory) were also included to PCA: lignocellulosic composition reported of the pretreated matter, lignocellulosic components losses compared to NIC, fungal biomass amount (fungi on the PCA) and matter yield considering no mass losses for control. The PCA concerned samples pretreated with different strains (identified with their BRFM number on PCA plans: Figure 7-9, Figure 7-10 and Figure 7-11) during three weeks and two samples pretreated two weeks (1554-2w and 985-2w) as it was the case for pyrolysis study (Chapter 5). Except non dimensionless variables, all variables (Data table in Annex 17) are expressed per amount of pretreated VS (for BMP) or TS (other variables). Expression of BMP in VS is relevant because only organic matter is used during anaerobic digestion. The comparison with variables expressed in TS is possible since BMP in pretreated VS are correlated to the BMP in pretreated TS (Annex 18) due to similar VS amounts (/g TS) between samples.

Simca software considered three axes for this PCA. In the plan defined by axis 1 and 2, 73% of the information contained in data can be found (Figure 7-9). The proximity between yield and lignin in the plan 1-2 shows that the more lignin remained the less matter was attacked; and obviously, the losses were lower since lignin (LIG), cellulose (Cel) and hemicelluloses (Hemi) losses are anti-correlated to lignin and yield variables. As previously observed for selection and optimization steps, LIG, Cel and Hemi losses compared to NIC are also correlated (Figure 7-5 and Figure 7-6), it can show the **impossibility to consume only lignin for fungi** (Kirk and Farrell, 1987). In Figure 7-9, fungal biomass amount (Fungi) is linked to TKN, notably because it is mainly constituted by proteins (Chapter 6). Fungi protein content can be approximated using TKN analysis (Li et *al.*, 2001) but a part of TKN results is due to chitin (glucose-like fungal polymer) (Díez and Alvarez, 2001). Thus, fungal protein amount determination with TKN is not precise. Increased in TKN content is linked to fungal biomass and fungal biomass improves slightly BMP (Chapter 4). The **correlation between BMP and Fungi is slight** as the one between DML and BMP for samples from the optimization step (DML was supposed related to fungal biomass amount).

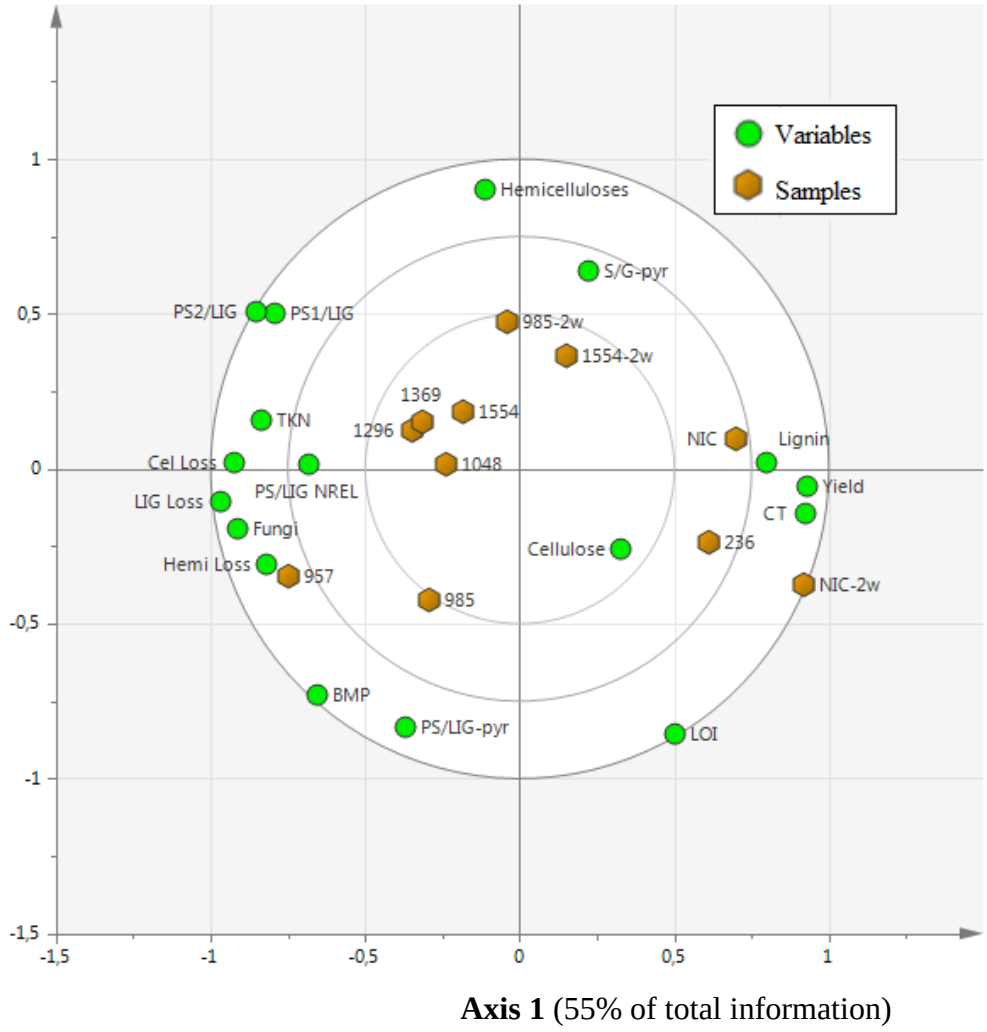


Figure 7-9. Plan 1-2 of PCA for characterizations of pretreated staws with several strains.

Moreover, with samples from the selection steps, it was highlighted that the use of different fungal strains with different compositions (and different BMP) limits the potential correlations (Figure 7-5D). TKN content is not linked to BMP probably because nutritive medium used for BMP tests provides all necessary nitrogen to microorganisms. LIG, Cel and Hemi losses are also correlated to TKN and anti-correlated to TC. It reflects the **decrease of C/N** (Annex 13) by consumption of carbon and conservation of nitrogen in fungal pretreated samples. High carbon contents correspond also to high lignin contents and high matter yields. In those conditions, BMP have a tendency to decrease but it is little correlated to lignin content in the plan 1-2. Hemicelluloses content is poorly correlated to BMP. The slight anti-correlation between hemicelluloses and BMP may be due to an increased recalcitrance of remaining hemicelluloses (as observed by others after fungal pretreatment, Chapter 1, 1.6.6.2). Concerning cellulose, it is not well represented in the plan 1-2 ($||\text{cellulose}|| \leq 0.5$), thus it cannot be discussed. A correlation exists between BMP and PS/LIG determined with pyrolysis (as observed in Chapter 5). For straws pretreated with different strains, the more PS/LIG-pyr is high, the more BMP of the substrate increases. PS/LIG determined with NREL method (acid hydrolysis) is slightly correlated to BMP but the quality of representation of PS/LIG NREL is poor. If PS1/LIG and PS2/LIG both determined with FTIR are highly correlated, they are independent of BMP variable in the plan

1-2. They are opposed to LOI, it can reveal a low contribution of crystalline cellulose to PS1/LIG and PS2/LIG. LOI is poorly or not linked to other variables. Its slight link with PS/LIG-pyr would perhaps indicate the higher contribution of crystalline structure in PS determined with pyrolysis. Crystallinity of cellulose can influence cellulose behavior under pyrolysis (Zhang et *al.*, 2010). Amorphous regions are earlier attacked than crystalline ones under pyrolysis (Zhu et *al.*, 2004), thus they are probably more degraded and lead to less characteristic fragments. S/G-pyr is anti-correlated to BMP whereas the contrary is expected (Monlau et *al.*, 2013). It was already explained that S/G-pyr variations are small among samples (Chapter 5). Rather than representing lignin structure, high S/G-pyr would be more reflective of high lignin amount determined with pyrolysis that is anti-correlated to BMP (through the ratio PS/LIG-pyr).

Concerning samples representation, there are all well represented in the space defined by the three components and in resulting plans since $Dmod_X$ values are below the critical value (Dcrit) (Annex 19). Samples 957 and 985 are the most linked to BMP, these two strains effectively improved BMP (NmL/g pretreated VS) of pretreated straws. Sample 957, corresponding to straw pretreated with BRFM 957, is also the most degraded sample (high losses) and the one with the highest fungal biomass as it is well shown on the plan1-2. NIC, 236 and 2-weeks pretreated samples are situated on the right of axis 1 (except 985-2w in the middle). They correspond to no or little degraded samples (close of high yield and lignin, low BMP and fungal amount). Sample 236 corresponds to straw pretreated with the Brown-Rot Fungus (BRF), *G. trabeum* BRFM 236. BRF are known to attack carbohydrates and to little modify lignin, it is logical to observe less degradation for 236 as it is shown by the proximity with NIC on the plan 1-2. The comparison between samples pretreated two weeks and three weeks shows that **two weeks are not enough to obtain an efficient pretreatment** since 985-2w and 1554-2w are closer to NIC samples and farther from BMP variable. A direct comparison of BMP is not possible since they were measured using different anaerobic inocula. Moreover, samples pretreated two weeks were inoculated with 10 mg $TS_{\text{fungal biomass}}$ more than those pretreated three weeks. For those reasons, BMP of samples pretreated two weeks were not included in plotted PCA (Data table in Annex 17). The lowest BMP obtained after pretreatment of two weeks compared to three weeks was largely discussed for BRFM 985 (Chapter 4). It also seems that two weeks pretreatment is too short with the strain BRFM 1554. Finally, it can be noticed that all samples have a medium value for LOI, except NIC-2w that seems to have a high LOI (Figure 7-9, Annex 17). 236, NIC and 985 have a LOI slightly higher than other samples. The lack of diversity in data may explain why BMP and LOI are not correlated on plan 1-2.

In the plan defined by axes 1 and 3 (Figure 7-10), 66% of total information is represented, and new correlations appeared compared to plan 1-2. Various known relations (from literature or current work) between BMP and chemical composition can be observed (Figure 7-10): PS/LIG NREL is correlated to BMP (as observed in Chapter 5), lignin is anti-correlated to BMP, and cellulose amount is poorly but positively correlated to BMP. For plotted samples, increase PS/LIG corresponds to BMP improvement and to a decrease lignin amount. Moreover, a slight tendency to BMP improvement corresponds to high

cellulose amounts. Some existing relationship in the plan 1-2 still exists in the plan 1-3, such as positive correlations between CT and Yield; between Cel, Hemi and LIG losses, Fungi and TKN and between PS1/LIG and PS2/LIG. Interestingly, **PS1/LIG** is rather positively related to BMP and rather negatively correlated to lignin, and **may give interesting information to study anaerobic biodegradability of pretreated straws** (contrary to PS2/LIG). In the plan 1-3 pyrolysis data, LOI and Hemicelluloses are not well represented.

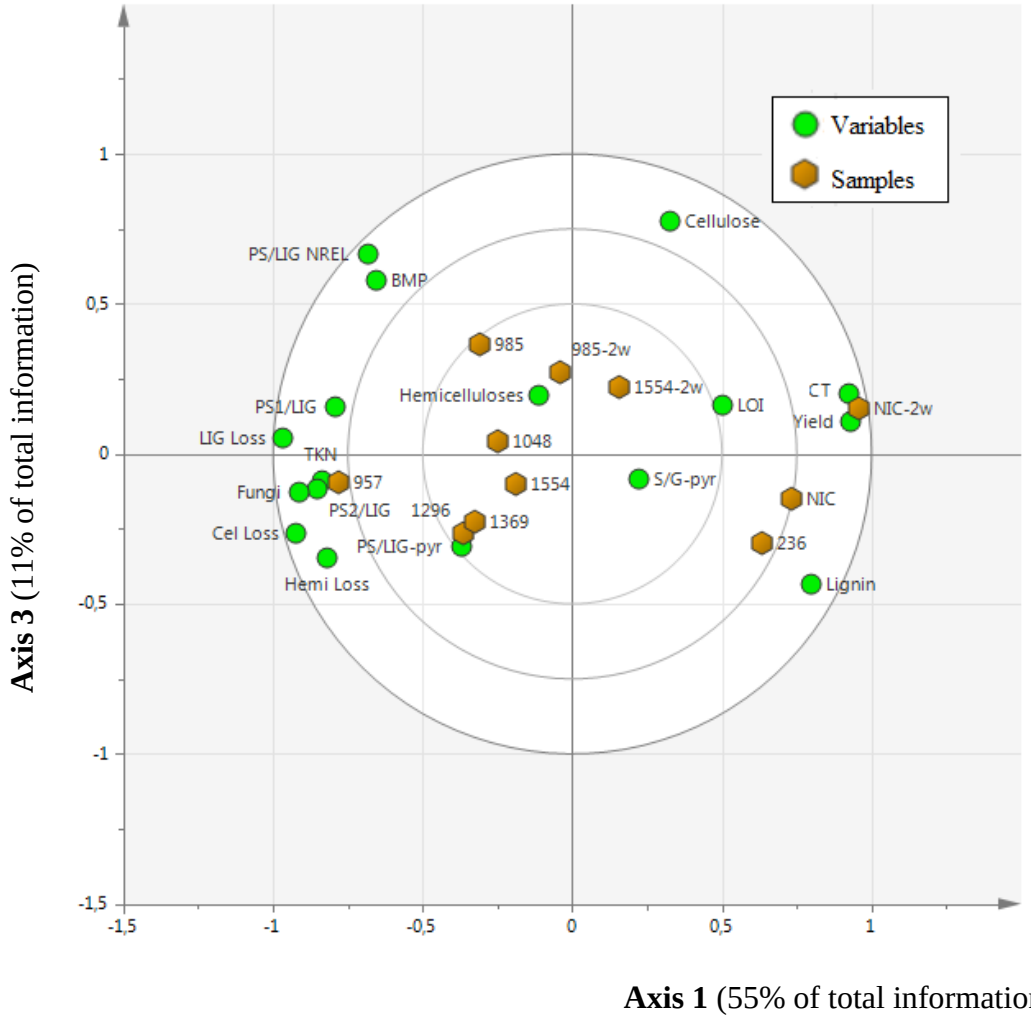


Figure 7-10. Plan 1-3 of PCA for characterizations of pretreated staws with several strains.

In the plan 2-3 (Figure 7-11), 29% of the total information can be found. In this plan, sample 985 is highly linked to the BMP variable, this sample corresponds to *Polyporus brumalis* and leads to the best methane improvement observed in this PhD thesis. There is a positive correlation between cellulose and PS/LIG NREL that reflects the higher contribution of cellulose in PS/LIG NREL since cellulose is present in higher amount in straws (pretreated or not) compared to hemicelluloses (Annex 17). Hemicelluloses and PS/LIG-pyr are anti-correlated that may reflect the absence of compounds representative of hemicelluloses in pyrolysates (Chapter 5), notably owing to the facility to degrade this compounds and thus to product unspecific compounds with pyrolysis.

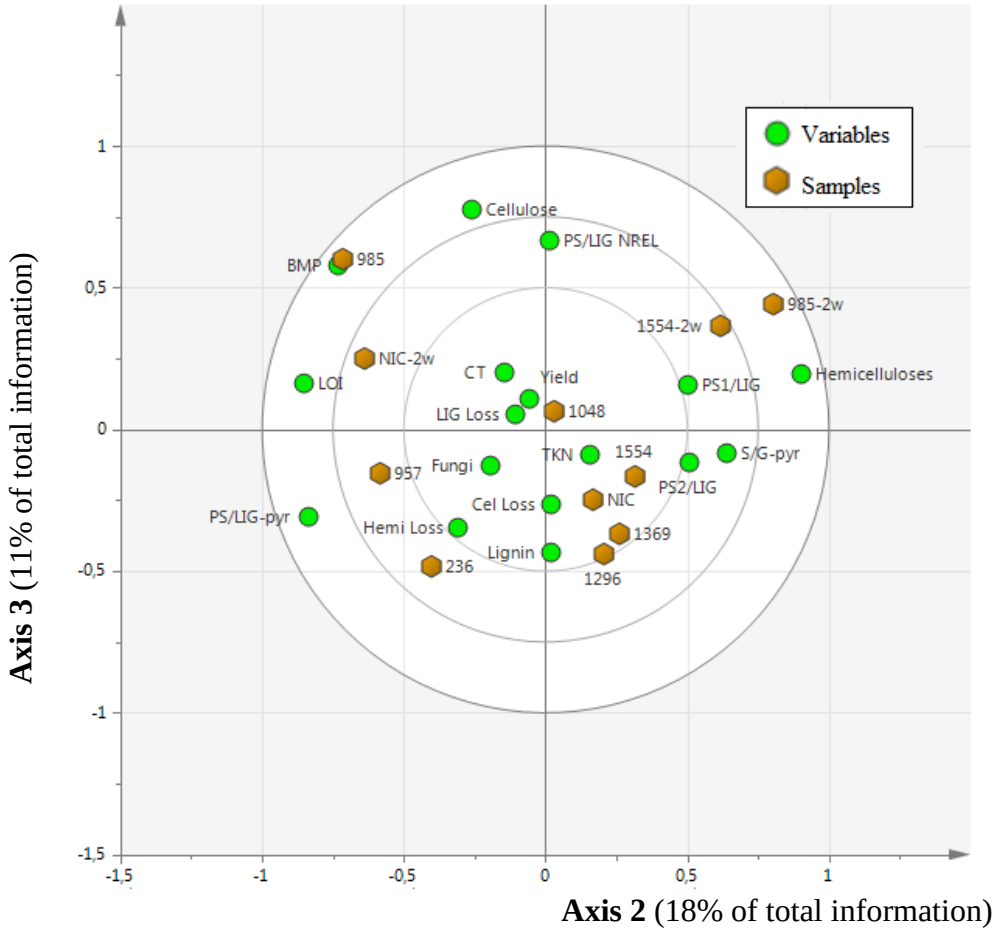


Figure 7-11. Plan 2-3 of PCA for characterizations of pretreated staws with several strains.

The LOI is positively correlated to BMP whereas the contrary is expected (according literature). The lack of variability between samples was already evoked but the measurement of cellulose crystallinity index with FTIR is perhaps also not relevant. Cellulose crystallinity index is usually measured with X-Ray Diffractometry (XRD) but the simplest and most affordable method to assess it is using FTIR. FTIR only provides relative values since spectra always contain contributions from both crystalline and amorphous regions. However, many studies compared successfully FTIR and XRD cellulose crystallinity (Park et *al.*, 2010). To better understand the effect of fungal pretreatment on cellulose crystallinity, four samples were measured by A. Barakat from the IATE UMR (project partner) with the XRD technic (Table 7-3). Monlau et *al.* (2012b) succeeded to correlate Crystalline Index determined with FTIR and with XRD on diverse lignocellulosic biomass. Using the same methodology (except grinding technic) as Monlau et *al.* (2012b), no correlation was found between FTIR and XRD measurements for measured samples. The difference in grinding method can perhaps explain the absence of correlation since grinding can change cellulose crystallinity (Mihranyan et *al.*, 2004). The small number of samples for XRD measurements is another possible explanation for the absence of correlation. However, crystallinity index was higher for NIC than for measured fungal treated samples (Table 7-3), it confirms the fungal capacity to attack crystalline cellulose reported in literature (Chapter 1). It can be noticed that *P. brumalis* BRFM 985 allowed to reduce crystalline

cellulose by half (%TS) compared to NIC. This capacity had contributed to the efficiency of this strain for ethanol or biogas production. As expected, fungal pretreatment decrease cellulose crystallinity (Table 7-3). Only few data are available in literature concerning modifications of cellulose crystallinity by WRF pretreatment (Chapter 1, 1.6.2). XRD measurements show that **important decrease of crystallinity** can be obtained with WRF pretreatment even without metals addition (shown efficient in literature), it was not demonstrated to our knowledge.

Table 7-3. Crystalline cellulose measured with XRD and FTIR for few samples from the selection step.

	Crystallinity Index or CrI (% TS Cellulose)	Crystalline cellulose with DRX (%TS)	Crystalline cellulose with FTIR (%TS)
T. ljubarskii BRFM 957	26.85	14.45	20.09
T. cingulata BRFM 1296	39.70	14.89	18.68
P. brumalis BRFM 985	36.27	10.28	21.62
NIC	51.09	20.84	21.95

More samples would have helped to find significant correlations in the PCA (e.g. for S/G) and perhaps to build a predictive model. At the LBE, straws issued from optimization step were only used for BMP tests and few samples were measured in pyr-GC-MS. Further characterizations of these straws were not possible due to the limited available amount. Taking into account 20% mass loss, around 16 g were available per tested condition. This amount was shared between different partners (4.5 to 6 g for the LBE). Moreover, one month and a half was necessary to pretreat all samples for optimization step at BBF lab by S. Zhou.

7.5 Other experimental prospects

Lignin amount is a good indicator of lignin degradation but cleavage of bounds between lignin and carbohydrates would be sufficient to improve biodegradability (Chapter 1). Thus, **study of lignin structure** can be interesting to explain anaerobic digestion of a substrate. Moreover, it can be useful to follow fungal degradation such as cleavage of ester links and consumption of ferrulic (FA) and p-coumaric acid (PCA). Diverse technics exist to study lignin structure such as NMR or a methylation step before py-GC-MS (Chapter 5) but they were difficult of access in the current work and project. However, a two-step protocol could have been used. **Amount of ester bonds** (links inside hemicelluloses) can be assessed after 24h at 25°C in a 1M NaOH solution; then, 2h at 170°C in 4M NaOH is required to assess ether links (links between hemicelluloses and lignin and inside lignin). FA and PCA ester-linked are generally lower in fungal treated substrate than in untreated control (Akin et *al.*, 1993b). Phenolic compounds are then identified and quantified with GC-MS (Yosef and Ben-Ghedalia, 2000) but quantification can also be assessed using coloration with Folin-Ciocalteu and spectrophotometry (Boizot and Charpentier, 2006). This quite simple analysis can be envisaged in further studies.

In literature review (Chapter 1), the difficulty for anaerobic microorganisms to **attack epidermis cells** in plant was exposed. It is notably due to a lipid layer (cutin, waxes). In contrast, fungi can degrade this layer. Thus, lipids released by fungal attack can present an interest to follow fungal degradation for anaerobic digestion. Such a study would have been possible with Py-GC-MS but with another kind of column that the one used in Chapter 5 (that was polar whereas apolar one is required). Moreover, an extraction step can be necessary to better visualize minor compounds of plants epidermis (Nip et *al.*, 1986).

Porosity is another important parameter impacting BMP that can be modified by fungal pretreatment. Some porosity measurements are in process at IATE UMR (A. Barakat, STOCKACTIF partner). Fungi increase the porosity of plant cells by the hyphal penetration and notably the attack of epidermis cells. The usual degradation from the center (vacuole) to the outer during subsequent anaerobic digestion is possibly modified due to new accessible surfaces. A digestion with ruminal microorganisms observed under microscope seems to confirm this assumption (Akin et *al.*, 1995). Anaerobic digestion of wheat straw was studied at a microscopic scale and it was concluded that substrate accessibility is the main limitation factor of SSAD degradation (Motte, 2013). To our knowledge, **microscopic observations of anaerobic digestion of a fungal treated substrate** have not yet been realized. It would allow checking if outer surfaces can be attack from the beginning of anaerobic digestion of fungal pretreated substrate.

Impact of fungal pretreatments can also be evaluated regarding their influence on **quality of digestate** issued from anaerobic digestion of a WRF pretreated substrate but further studies are required to answer this problematic. As WRF can also be used for their depolluting role towards aromatic molecules, they would improve digestate quality in this way. Besides *P. brumalis* is known to degrade micropollutants such as dibutyl phthalate (DBP), this kind of pollutants has a huge environmental impact (Pinedo-Rivilla et *al.*, 2009). In the current study, major fertilizing elements (N, P, K) of digestate were not fully studied after SSAD in batch since it was not the goal of the study. Moreover, pretreatment was not optimal and would not allow a sufficient characterization of fungal pretreatment impact on digestate. No difference in nitrogen content was found on solid digestate after fungal pretreatment (Chapter 6). Increase of nitrogen amount was found in leachate but they are generally recirculated whether than spreading in fields. In general, there is a lack of knowledge about digestates impacts on the environment or human health and there is even a debate about their effectiveness as amendment or fertilizer (Nkoa, 2014).

Spectrophotometry with Folin-Ciocalteu and 3D-fluorescence spectroscopy can be used to characterize clean-up water from fungal treated straws, especially phenols issued from lignin degradation. It would allow to investigate fungal degradation of lignin and to study an eventual link with BMP of the substrate. Moreover, phenols can have a high commercial value. A better knowledge of **phenols easily released by fungal pretreatment** is required for their potential valorization. Phenols could be a co-product of fungal pretreatment. 3D-fluorescence spectroscopy allows the visualization of fluorescent compounds (aromatic) such as those contained in protein, lignin and phenols (Jimenez et *al.*, 2015). It provides notably some information about the polymerization degree of molecules in extracted samples. Spectrophotometry with

Folin-Ciocalteu allows to estimate total phenolic content (Boizot and Charpentier, 2006). Diverse project partners were concerned by phenols availability experiments namely, LBE, Envolure company and BBF. Some characterizations are still in process and expected to draw conclusions on this point.

7.6 Conclusion

In summary, taking into account mass losses more than 20% methane improvement compared to control straw were obtained with *P. brumalis* BRFM 985. It is very possible that even greater value can be obtained. To our knowledge, it is the best improvement observed on pretreated grasses and straws but there are very few studies available to compare. It cannot be excluded that slight starter addition allowed such an improvement. Notwithstanding, *P. brumalis* BRFM 985 seems to have very interesting properties. Further investigations are required to improve SSAD of fungal pretreated straws (not only on fungal pretreatment but also on SSAD processes). For both optimization and selection steps, there were a correlation between BMP and cellulose digestibility and a slight positive influence of fungal biomass on BMP. Carbohydrates losses were generally correlated to lignin losses. Finally, important decreases of cellulose crystallinity were measured on few pretreated samples from the selection step.

Chapter 8. Expanded prospects about aerobic fungi and anaerobic digestion and conclusion

Difficulties encountered during the STOCKACTIF project, proposed improvements and additional experimentations were already discussed, especially in the previous chapter. However, in a broader context diverse prospects can be considered for fungal pretreatments.

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8.1 Combination of pretreatments

White-rot pretreatment in combination with physical or chemical pretreatments can reduce the harshness of the pretreatment leading to environmental and economic gain compared to pretreatment without WRF. Moreover, the long period required by fungi would be reduced. Pretreatments combination is little investigated for anaerobic digestion application but some studies for bioethanol application were reported. They can furnish useful information for further study of anaerobic digestibility (BMP), e. g. delignification with low sugar loss may lead to increased BMP. Effects of some pretreatments combination on the composition of resulting lignocellulosic biomass are resumed in Table 8-1. Good results were reported for fungal treatment followed by NaOH, acid or steam explosion step and also for H_2O_2, hot water and ultrasound treatment followed by fungal step (Wan and Li, 2012). A two-step fungal and alkali treatment led to an increased dry mass loss (more reactive sites) but it was compensated by an increased conversion of cellulose to glucose. Accessibility to cellulose was improved because fungal pretreatment facilitated the removal of hemicelluloses and lignin during alkaline fractionation. Thus, a synergetic effect occurred on cellulose digestibility (Yang et *al.*, 2013).

Table 8-1. Combined white rot fungal pretreatment and physical/chemical pretreatment.
(Adapted from Wan and Li, 2012)

Fungal pretreatment	Physical/chemical pretreatment	Substrate	Effectiveness
Prior [a]	Alkaline (NaOH)	Corn stalk	Lignin *and xylan removal*
Prior	Diluted acid	Water hyacinth	Sugar yield
Prior	Steam explosion	Beech wood meal	Sugar yield
Post [b]	Alkaline (NaOH)	Wheat straw	*Strong alkaline pretreatment masked the synergistic effect of fungal pretreatment*
Post	H_2O_2	Rice hull	Acceleration of fungal delignification
Post	Ultrasound	Beechwood and pine sawdust	
Post	Ultrasound	Rice hull	Acceleration of delignification *with slight sugar loss*
Post	Hot water extraction	wheat straw	
Post	Liquid hot water pretreatment	Soybean straw	Delignification
Post	Acid (Sulfuric acid)	Beechwood and pine sawdust	
Post	Alkaline (Ammonia)	Beechwood and pine sawdust	Reduce lignin degradation on fungal pretreatment

0

[a] Prior indicates that fungal pretreatment is the first step of combined pretreatment.
[b] Post indicates that fungal pretreatment is the subsequent step of combined pretreatment.

8.2 Use of other substrates

In literature, diverse lignocellulosic biomasses were pretreated with WRF for anaerobic digestion. A particular efficiency on woody biomasses is reported (Chapter 1, Table 4-3). Wood has low BMP value and is little used in anaerobic digestion plants but with fungal pretreatment new substrate such as sawdust would be usable.

The improvement of anaerobic digestibility of manure and/or spent straw (bedding material) using WRF would be of great interest. High microorganism amount in such substrates could constitute a difficulty for

pretreatment in non-sterile conditions. It was reported that urea on forage is converted to ammonia by autochthonous flora. This phenomenon leads to pH increase resulting in the inhibition of many putrefying organisms. WRF able to grow at elevated pH such as *Coprinus* fungi can consequently delignify the substrate in the absence of putrefying organisms (Krause et *al.*, 2003). Horse manure, in particular, contains great quantity of bedding material (generally straw) and have a low fertilizing value which predestines it to the anaerobic digestion alternative. With SSAD, it was shown that spent straw from horse stall was more biodegradable than raw wheat straw (Cui et *al.*, 2011). With a full-scale wet plant (continuous process), anaerobic digestion of horse manure without pretreatment was not feasible for practical application: low degradation and increase risk of mechanical problem linked to high viscosity or floating layers (Mönch-Tegeder et *al.*, 2014b). Horse manure is difficult to store without uncontrolled degradations (and loss of subsequent methane production) that is why a WRF pretreatment would be interesting.

8.3 Mixed culture and strain engineering

One of the difficulties to study fungal pretreatment lies in multiple interactions between fungal strain, substrate and culture conditions. Thus, it would become harder using combination of two or more strains belonging to BRF and WRF. Notwithstanding, such combinations can be synergistic on pretreatment. An enhanced ligninolytic activity was observed with the combination of *P. brumalis* and *T. versicolor* on synthetic agar medium (Sundman and Näse, 1972). With SSF on oat straw, fungi in pair-wise combinations were found to slightly improve cellulose digestibility of pretreated substrate (Levonen-Munoz et *al.*, 1983). The synergistic effect can be caused by a complementarity of enzymatic materials. Another application can be envisaged combining a fast growing strain to limit contamination and a selective delignifying one to pretreat efficiently the substrate.

Another possibility to increase fungal pretreatment efficiency is the recourse to genetic transformations. Overexpression of the laccase gene from *P. brumalis* lead to 1.6-fold higher sugar yield than with wild strain after the hydrolysis of pretreated wood chips (Ryu et *al.*, 2013). However, increased knowledge would be required. A central role of MnP enzyme in delignification is highlighted but diverse conclusions can be found in literature concerning best enzymatic equipment to delignify and lignin selective attack is rarely considered (Hatakka, 1994; Moreira et *al.*, 1997). Furthermore, possible risks for the introduction of genetically modified organisms in the environment, such as impact on biodiversity and ecosystem function, are not fully assessed (Wolfenbarger and Phifer, 2000). The whole natural existing potential of WRF strains is still not completely explored. Thus, this exploration would be preferable to strain engineering.

8.4 Fungal culture on digestate

Residual organic matter in digestate and the resulting biomethane can be economically attractive (Singh et *al.*, 2007). An original study failed to obtain more biogas by treating lignocellulosic digestate (from wheat straw, corn stover or grass) with WRF. The goal was to degrade carbohydrates with anaerobic digestion and then, to alter the remaining lignin with WRF in order to digest it anaerobically again (López et *al.*, 2013).

Digestates are rich in NH_4-N (Singh et *al.*, 2007) and N starvation is generally required for delignification by WRF. Thus, it is possible that too available nitrogen had limited the delignification of the digestate by WRF. Fungi from other phylum (Ascomycota) (Singh et *al.*, 2007) could be more able to degrade lignin in digestate.

It seems possible to treat digestate with ligninolytic fungi when the goal is a reduction of waste amount (without energetic valorization). López-Abelairas et *al.* (2013) obtained a reduction of around 45% for resulting hemicelluloses. After a centrifugation step, around 30% VS degradation were observed on autoclaved digestate of kitchen refuse with fungal pretreatment with the soft-rot *Chaetomium cellulolyticum* or with the WRF *P. chrysosporium*. Best lignin reductions in digestate were between 29% (for *P. chrysosporium* BKM YM 125) and 45% (for *C. cellulolyticum*) (Schober and Trösch, 2000). This lignin reduction may lead to increase residual methane production but it was not studied.

With another goal than delignification or methane production, it was suggested to use certain fungi able to produce lipid in order to produce biodiesel, those fungi would be able to grow on digestate (Zhong et *al.*, 2015). Fungal lipids are likely to increase the BMP of fungal biomass but production of biodiesel with them is likely more remunerative than methane production.

8.5 Biohydrogen production

Hydrogen which is not at present commercialized as an energy source but widely used as a chemical reactant, can be produced biologically with dark fermentation. This process is similar to anaerobic digestion but with an inhibition of methanogenesis. The metabolic pathways leading to hydrogen production must also be favored. However, dark fermentation also requires an efficient hydrolysis step and the problem of lignin recalcitrance must be circumvented (Guo et *al.*, 2010). WRF pretreatments can be interesting to improve biohydrogen production. Zhao et *al.* (2012) used a pretreatment of cornstalks with *Phanerochaete chrysosporium* to enhance enzymatic saccharification and hydrogen production.

8.6 Production of edible mushrooms

One way to increase the economic efficiency would be the cultivation of edible WRF (like *Pleurotus*) concomitantly to the delignification process followed by energy valorization. Mushrooms are more valuable (Tsang et *al.*, 1987) and delignification can be used to diminish cultivation wastes. The resulting energy production can be used, for example, to maintain a good temperature for mushrooms cultivation. The mycelial colonization allowing delignification and the formation of edible fruiting bodies are different stages in the life cycle with lots of functional differences (Kalmış and Sargın, 2004). The concomitant optimization of these two mechanisms is difficult. Authors who tried, obtained poor results regarding the delignification or *in vitro* degradation of spent substrates (Tsang et *al.*, 1987; Zhang et *al.*, 2002).

8.7 Other applications of WRF

Possible applications for WRF are huge: sewage treatment, biopulping, conversion to animal feeds, compost inoculants, production of medicinal mushrooms (Isroi et *al.*, 2011; Tian et *al.*, 2012). Increase knowledge on WRF mechanisms can thus sometimes serve several applications. Finally, some more original uses are reported such as coal extraction after its liquefaction with *T. versicolor*. After drying *Pleurotus tuber-regium* can even be a weapon (bludgeon) as reported in Papua New Guinea (Fourré et *al.*, 1990).

8.8 Conclusion

To face current global challenges, economic growth must not be at the expense of the environment and of the human being. With this in mind, second generation biorefineries based on non-edible lignocellulosic substrates are necessary. The costly pretreatment step required to sufficiently valorize those substrates, is currently a major limitation to the development of second generation biorefineries. Consequently, pretreatments represent huge commercial and industrial opportunities, as it can be attested by recent patents on lignocellulosic pretreatments (Bule et *al.*, 2013; Gimbert et *al.*, 2014).

In this context, white-rot fungi pretreatments, requiring little inputs, arise a growing interest. Diverse studies investigated the possibility for WRF to improve cellulose hydrolysis for ethanol application. However, WRF were little investigated for anaerobic digestion purpose, although it is a very efficient way to produce energy. Moreover, diversity of studied strains for pretreatment is weak (*Ceriporiopsis subvermispora*, *Pleurotus florida*, *Phanerochaete chrysosporium…).* Pretreatment conditions constitute a central point for the efficiency of a couple strain/substrate but they are often not discussed in WRF pretreatment for anaerobic digestion studies.

The current study investigated WRF pretreatments of wheat straw for anaerobic digestion. Firstly, there were two applied objectives consisting to select an efficient strain and to optimize its culture conditions. The common goal consisted to maximize methane production of pretreated straws. After a screening step, *P. brumalis* BRFM 985 was chosen. For the first time, an attempt to optimize pretreatment conditions for anaerobic digestion was carried out. Best pretreatment conditions were not reached in the experimental domain but maximum values were observed with almost 25°C and 3.5 initial wet weight/TS. Metals addition during the pretreatment significantly enhanced methane production from pretreated straw, notably by increasing lignin losses. After metals addition, culture duration was the most impacting parameter. To obtain the maximum BMP values, ten days were required to pretreat the substrate without metals addition whereas twenty days were necessary with metals addition (with far better BMP values). Those results were obtained with BMP-tests that are not sufficient to predict full-scale performance. Thus, the third applied objective was to investigate fungal treated straw in batch Solid-State Anaerobic Digestion reactors. SSAD is particularly adapted for wheat straw valorization but during start-up phase, there is a high risk of acidification that can inhibit methanogenesis. However, it was shown that fungal pretreatment can facilitate the start-up of batch SSAD reactors (likely due to consumption of soluble compounds) and that substrate/inoculum must be between 2 and 3 (VS basis).

Scientific objectives were to quantify and to understand mechanisms implied by the fungal pretreatment of wheat straw in anaerobic digestion. Taking into account mass losses, more than 20% increase in methane yield were obtained in the best case. To our knowledge, it constitutes the best anaerobic digestion improvement for grasses or straws pretreated by fungi. In tested culture conditions, this improvement was notably due to the selective consumption of lignin by *P. brumalis* BRFM 985 along with a conservation of cellulose and a decrease of cellulose crystallinity. Such a selectivity seems primordial for pretreatment efficiency. Selectivity would notably decrease with glucose addition at the fungal inoculation time (in a strain dependent way, for a fixed culture duration and with at least 50 mg glucose/g straw). By contrast, metals addition increased the selectivity for lignin consumption.

The negative impact of lignin on methane production from lignocellulosic substrate was already known but this impact was observed even after only 6 days of BMP-tests. Improvement of methane production with fungal pretreatment would not be due to soluble sugars increase whereas the high degradability of fungal mycelium would improved methane production-rate in BMP-tests, especially during the first 30 days. Best calculated methane production during the optimization step, was obtained with 25% of dry matter losses and 30% of lignin losses (compared to lignin amount in NIC). It corresponded to 31% improvement (NmL/g TSi) compared to untreated wheat straw but only to 5% compared to non inoculated controls (NIC).

To finish, Py-GC-MS would allow to pre-select strains based on anaerobic degradability of fungal pretreated wheat straws since it allowed an estimation of fungal biomass amount. Moreover, polysaccharides/lignin ratios determined with Py-GC-MS were correlated to BMP of straws pretreated with several strains.

The necessity of adequate monitoring parameters for SSAD was also highlighted. In the current study, batch SSAD reactors were able to recover from acidification phase when TVFA/alkalinity was lower than 2 and with VFA concentrations inferior to 10 g/L in leachate.

To conclude, fungal pretreatments for anaerobic digestion were not yet fully exploited. There is notably a real need to increase the studied scale principally to quantify fungal pretreatment costs and to conclude about their industrial feasibility (such data are expected from the STOCKACTIF industrial trial).

Results from this work have highlighted fungal pretreatment potential and have raised awareness about: (i) the necessity to take into account mass losses during pretreatment to evaluate its efficiency; (ii) the importance to study pretreatment conditions in strain efficiency evaluation; (iii) the necessity to find ways to pretreat substrates under non sterile conditions.

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List of annexes

Annexes

Annex 1. Characterization of wheat straws in literature.
S. D.: Standard Deviation; G : Guaiacyl units in lignin ; S : Syringyl units ; H : p-Hydroxyphenyl; CrI: Crystallinity Index determined with DRX.

	Average	S. D.	Minimum	Maximum	Studies	References
TS *(% wet weight)*	93	6	77	98	9	(Motte, 2013)
VS *(% wet weight)*	84	8	63	93	9	
Ash content *(% TS)*	8	3	5	14	9	
Solubles *(% TS)*	12	5	5	18	9	
Cellulose *(% TS)*	29-35				3	(Sánchez, 2009)
Hemicelluloses *(% TS)*	26-32					
Lignin *(% TS)*	16-21					
Glucose *(% TS)*	36	3	4	11	6	(Adapted from Motte, 2013)
Xylose *(% TS)*	20	5	27	42	6	
Arabinose *(% TS)*	2	4	15	27	6	
Arabinose/Xylose *(%)*	9	0	2	3	6	
%C	47.2	3.5	41.8	53.6	6	
%H	5.5	0.3	5.1	5.9	8	
%O	36.7	3.8	31.4	41.6	8	
%N	0.6	0.3	0.1	1.1	7	
%S	0.1	0.06	0	0.1	3	
BMP Buswell *(NmL/g TS)*	466	35	420	536	8	
$BMP_{35°C}$ *(NmL/g TS)*	225	51	150	301	10	
$BMP_{55°C}$ *(NmL/g TS)*	265	6	261	272	3	
BHP *(NmL/g TS)*	5	4	0.5	9.5	3	
G/S/H	49/46/05				5	(Monlau et *al.*, 2013)
CrI *(% TS cellulose)*	50.3				5	
β-O-4 *(µmol/g lignin)*	1040				5	
p-coumaric acid *(mg/g TS lignin)*	77.4				1	(Dinis, 2009)
Ferulic acid *(mg/g TS lignin)*	57.5				1	

Annex 2. Cultural route of wheat straw used in this study.

* **Semis** du *07/10/2011* sur 3,49 ha

HAUSSMANN	145,131 kg/ha	507,174 kg	Blé tendre

* **Semis** du *08/10/2011* sur 3,01 ha

EXPERT	138,822 kg/ha	416,754 kg	Blé tendre

* **Herbicide** du *19/11/2011* *Cibles : Graminées+Dicotylédones annuelles*

CELIO	0,193 l/ha	1,254 l	Herbicide
BRENNUS	0,964 l/ha	6,268 l	Herbicide
MATIN	1,447 l/ha	9,402 l	Herbicide
ACTIROB B	0,723 l/ha	4,701 l	Adjuvants-Divers (avec AMM)
EPYLOG FLASH	0,289 kg/ha	1,880 kg	Fongicide

* **Amendement calcaire** du *28/02/2012*

Solution 39N	137,416 l/ha	893,201 l	54N

* **Roulage** du *16/03/2012*

* **Amendement calcaire** du *28/03/2012*

§ Solution 26N-17 S	200,000 l/ha	1 300,000 l	52N	34S

* **Fongicide** du *01/05/2012*

SPORTAK HF	0,501 l/ha	3,255 l	Fongicide
CHEROKEE	0,668 l/ha	4,341 l	Fongicide
MENARA	0,050 l/ha	0,326 l	Fongicide
MODDUS	0,250 l/ha	1,628 l	Régulateur
EPYLOG FLASH	1,102 kg/ha	7,162 kg	Fongicide
HELIOSOL	0,110 l/ha	0,716 l	Adjuvants-Divers (avec AMM)

* **Amendement calcaire** du *04/05/2012*

Solution 39N	203,673 l/ha	1 323,873 l	79N

* **Herbicide** du *07/05/2012*

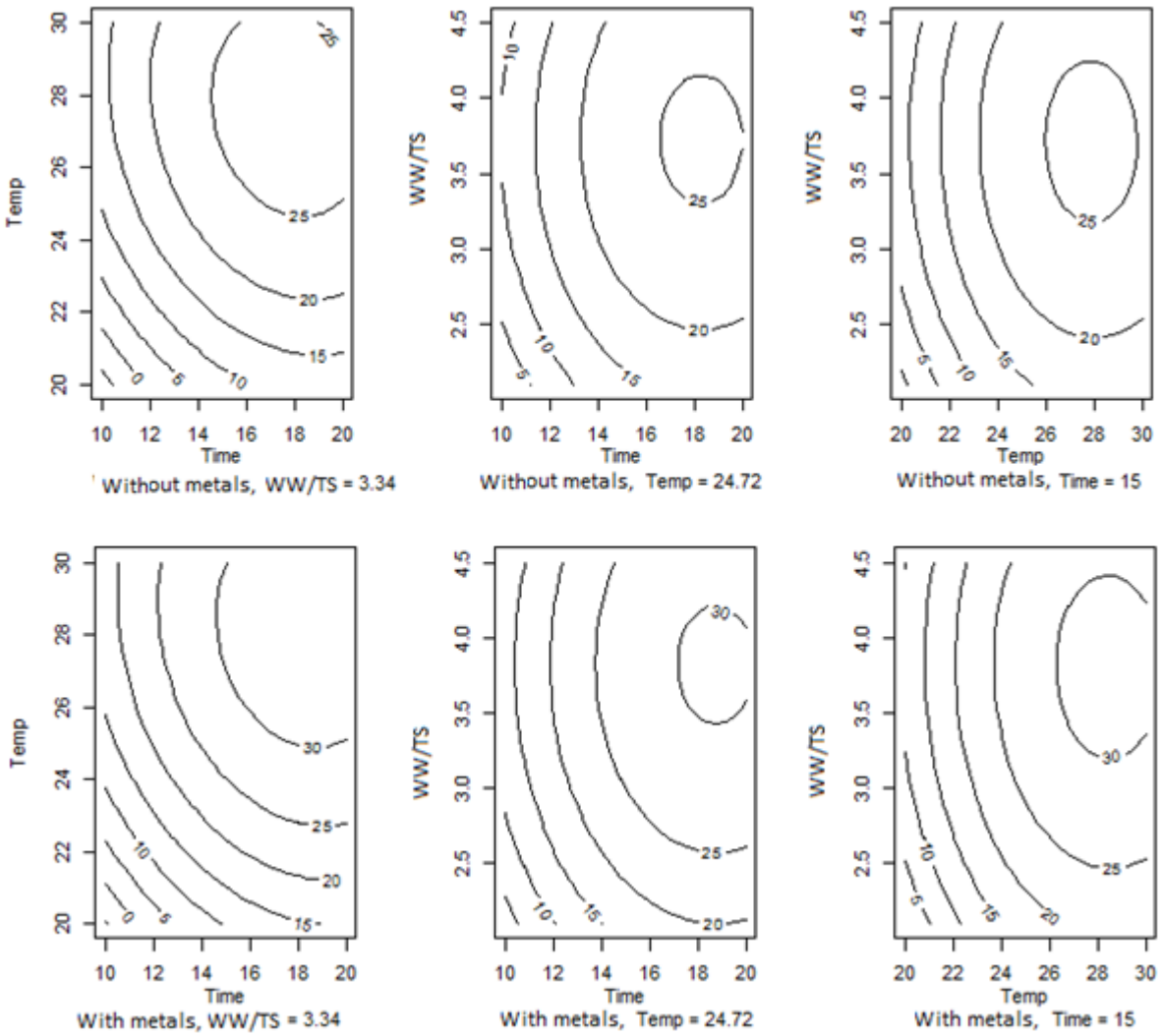
AGERZOL 1000	0,501 l/ha	3,255 l	Herbicide
ACTIROB B	1,002 l/ha	6,511 l	Adjuvants-Divers (avec AMM)
ALLIE STAR SX	15,025 kg/ha	97,663 kg	Herbicide
KART	0,501 l/ha	3,255 l	Herbicide
EPYLOG FLASH	0,835 kg/ha	5,426 kg	Fongicide
HELIOSOL	0,110 l/ha	0,716 l	Adjuvants-Divers (avec AMM)

Récolte du Blé : 25 juillet 2012

Pressage de la paille : 26 juillet 2012

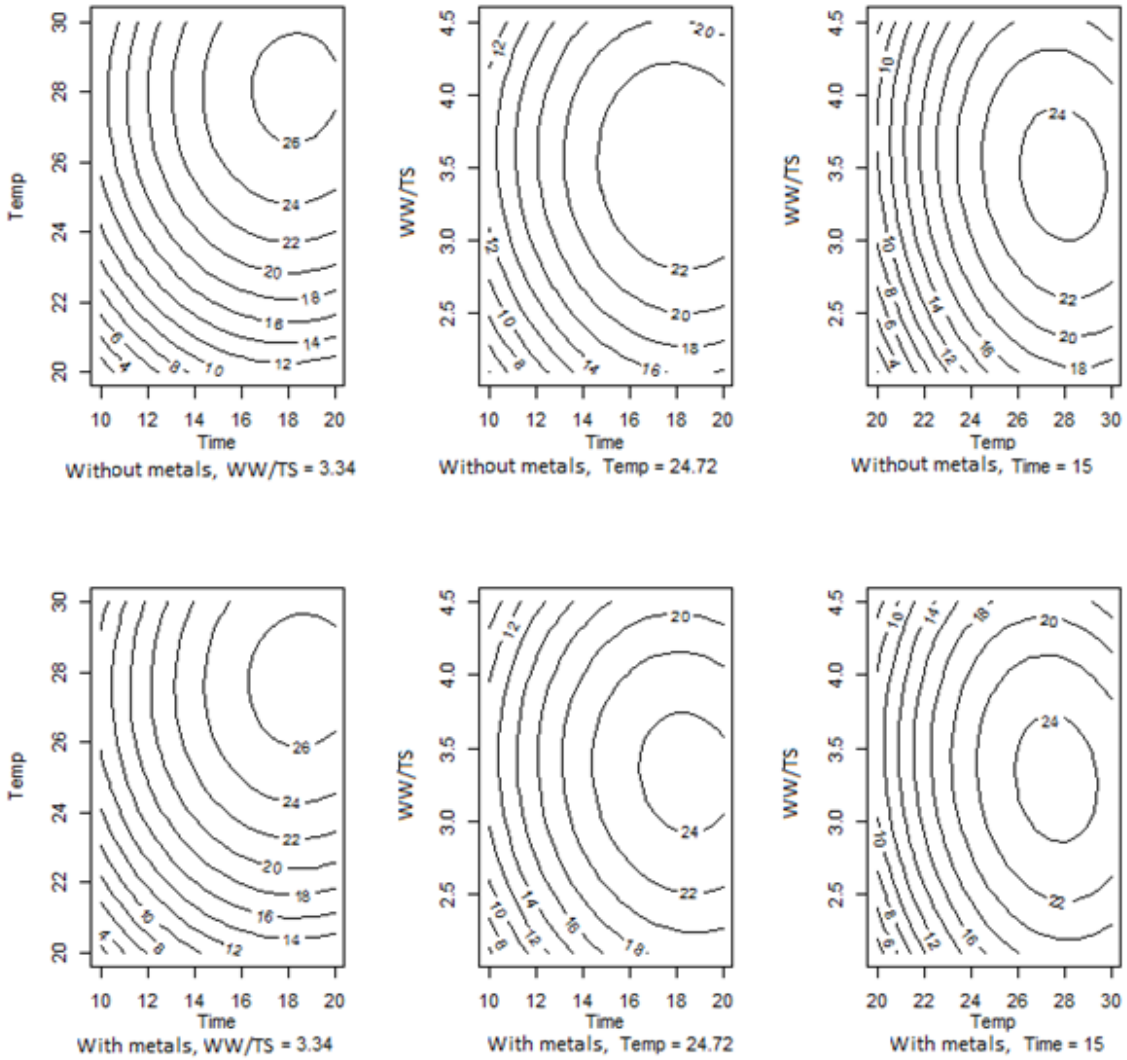
Annex 3. Response surfaces for lignin losses from optimization step.

Contour plots correspond to lignin losses (%TSi) compared to NIC depending on pretreatment parameters: Temp: Temperature in °C; WW/TS: initial moisture content in g/g; and Time: duration in days.



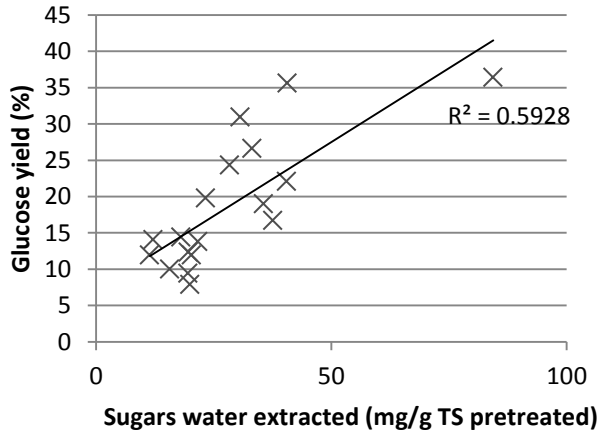
Annex 4. Response surfaces for dry matter losses (DML) from optimization step.

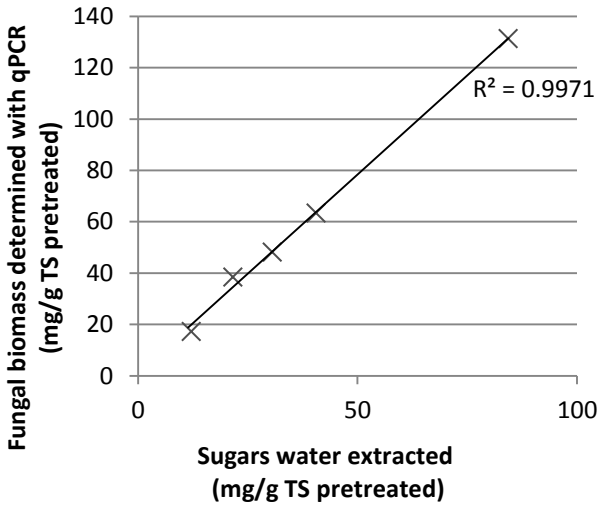
Contour plots correspond to DML (%TS) depending on pretreatment parameters:
Temp: Temperature in °C; WW/TS: initial moisture content in g/g; and Time: duration in days.

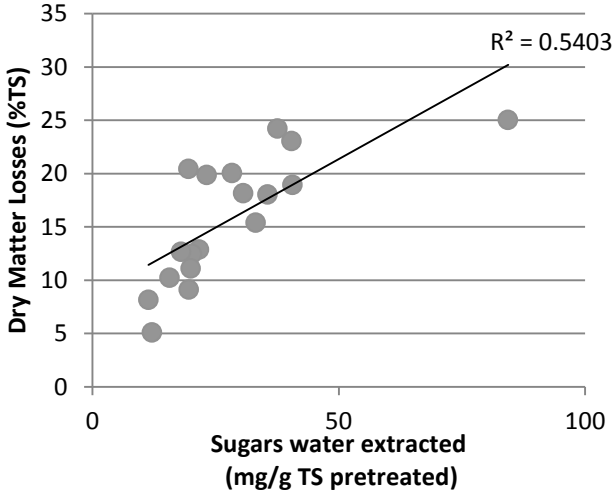


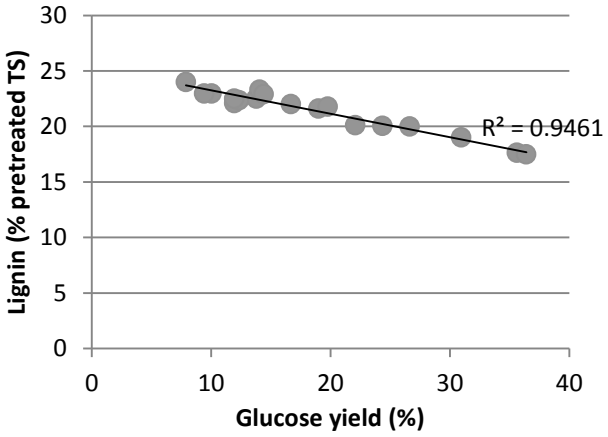
Annex 5. Table of model coefficients for studied response surfaces.							
		BMP *(NmL/g TS i)*	CH4 production at day 57 *(NmL/g TS i)*	CH4 production at day 6 *(NmL/g TS i)*	Lignin losses *(%)*	Glucose yield *(%)*	Dry Matter Losses (DML) *(%)*
b0	Constant	160.75	139.19	66.23	25.03	18.70	22.80
b1	Metals (met)	-14.42	-13.09	-2.79	-1.98	-3.40	-0.26
b2	Duration	1.51	2.57	4.76	7.75	3.12	5.50
b3	WW/TS	8.90	11.34	9.74	4.73	8.05	1.78
b4	Temperature	5.37	8.39	10.30	8.98	6.87	10.30
b21	Duration x Metals	-10.47	-5.53	0.25	-0.50	-1.26	-0.23
b31	WW/TS i x Metals	-2.75	-3.15	-3.14	-0.50	-0.37	0.96
b41	Temperature x Metals	-12.53	-11.65	-3.19	-0.81	-2.82	0.52
b22	Duration ²	25.60	19.56	-11.79	-5.55	0.26	-4.11
b33	WW/TS i²	-1.27	-7.88	-9.87	-6.98	1.58	-7.73
b44	Temperature²	-10.65	-9.32	-6.88	-6.98	-1.76	-8.55
b23	Duration x WW/TS i	-15.44	-11.33	-8.36	-0.03	1.16	-0.83
b24	Duration x Temperature	-16.64	-7.60	9.21	-1.97	3.42	0.46
b34	WW/TS i x Temperature	13.35	14.45	12.43	-0.27	10.85	-2.01

Annex 6. Relationships between soluble sugars and glucose yield after hydrolysis, dry matter losses and fungal biomass and correlation between lignin and glucose yield after hydrolysis for samples from the optimization step.



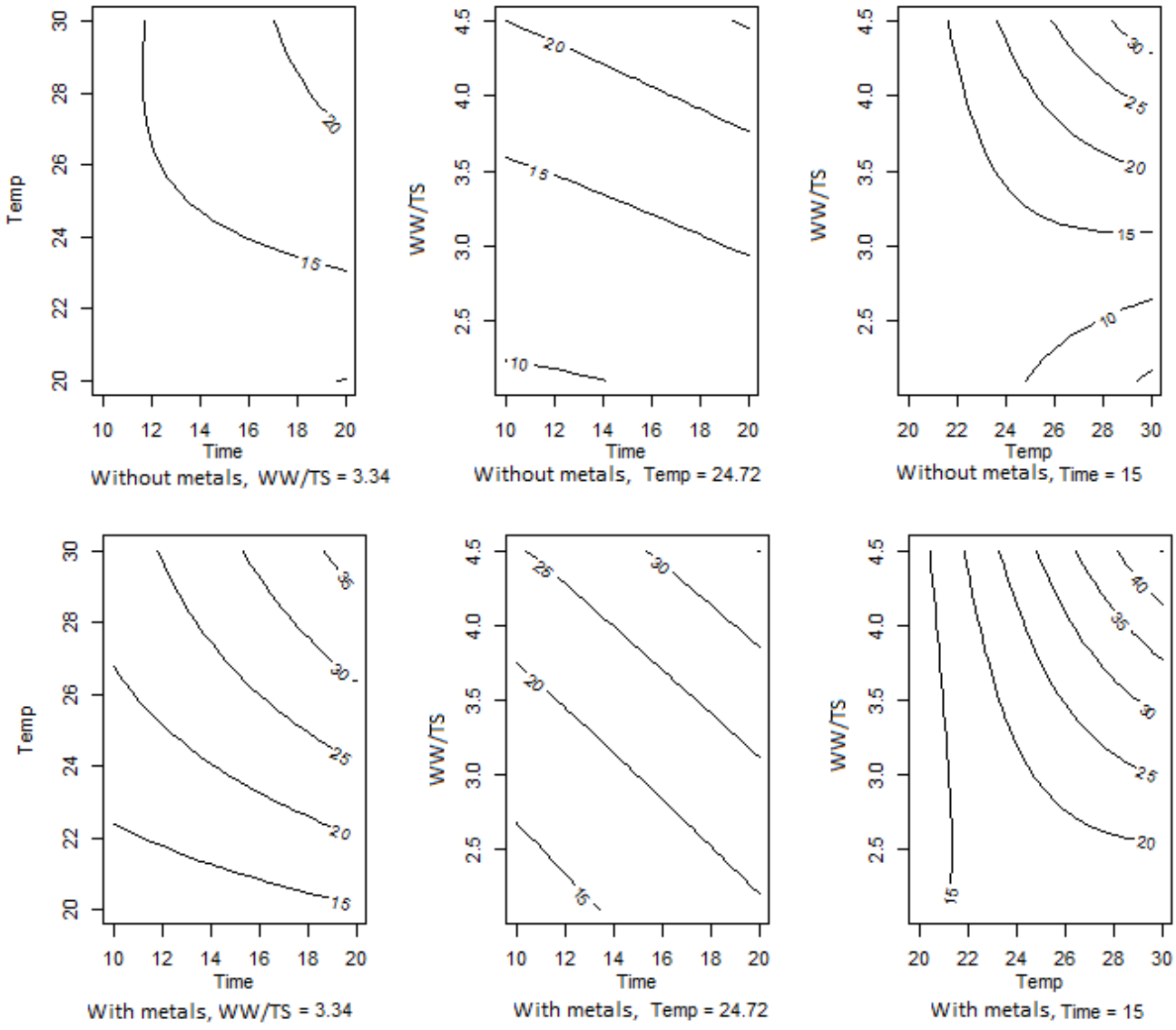






Annex 7. Response surfaces for glucose yield after hydrolysis from optimization step.

Contour plots correspond to glucose yield (%) depending on pretreatment parameters:
Temp: Temperature in °C; WW/TS: initial moisture content in g/g; and Time: duration in days.



Annex 8 (Part 1/2). Pyrolysis compounds of fungal pretreated straws and of *P. brumalis* BRFM 985: PS-class, LIG-class, N-class and UN-class.

PS: Polysaccharide origin; LIG: lignin origin; N: N-containing compounds; UN: Unspecific origin.

Code	Compound	Reference
PS3	2-Cyclopenten-1-one, 2-methyl-	(Dignac et *al.*, 2005)
PS6	Ethanone, 1-(2-furanyl)-	
PS7	2-Cyclopenten-1-one, 3-methyl-	
PS8	2-Cyclopenten-1-one, 2,3-dimethyl-	
PS9	2-Furancarboxaldehyde, 5-methyl-	
PS12	2-Furanmethanol	
PS16	1,2-Cyclopentanedione, 3-methyl-	
PS18	Levoglucosenone	
PS21	1,4:3,6-Dianhydro-.alpha.-d-glucopyranose	(Ross and Mazza, 2011)
PS24	1,6-Anhydro-.beta.-D-glucopyranose (levoglucosan)	(Dignac et *al.*, 2005)
PS25	Furfural	(Nowakowski and Jones, 2008)
PS26	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	(Ross and Mazza, 2011)
PS28	Furan, 2-methyl-	(Lv and Wu, 2012)
PS29	2(5H)-Furanone	
PS30	1,2-Cyclopentanedione	(Mészáros et *al.*, 2007)
PS31	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	(Nowakowski and Jones, 2008)
PS33	1,3-Cyclopentanedione, 2,2-dimethyl-	
PS34	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	(Nowakowski and Jones, 2008)
PS35	Furan, 2,5-dimethyl-	(Buurman et *al.*, 2007)
PS36	Maltol	(Pouwels et *al.*, 1987)
PS37	3-Furaldehyde	
PS38	2-Cyclopentene-1,4-dione	(Ross and Mazza, 2011)
PS40	2-Methyl-5-hydroxybenzofuran	
PS42	Benzofuran, 2-methyl-	(De la Rosa et *al.*, 2008)
PS43'	Anhydro-d-mannosan	(Nowakowski and Jones, 2008)
PS44	D-allose	(Ross and Mazza, 2011
PS46	1,3-Cyclopentadiene	(Pouwels et *al.*, 1987)

Annex (Part 2/2). Pyrolysis compounds of fungal pretreated straws and of *P. brumalis* BRFM 985: PS-class, LIG-class, N-class and UN-class.

PS: Polysaccharide origin; LIG: lignin origin; N: N-containing compounds; UN: Unspecific origin.

Code	Compound	Current name	Reference
LIG1-G	Phenol, 2-methoxy-	(guaiacol)	(Dignac et *al.*, 2005)
LIG2-G	Phenol, 2-methoxy-4-methyl-	(Me-guaiacol)	
LIG4-G	(2-Methoxy-4-(2-propenyl)-phenol)	(eugenol)	
LIG5-G	Phenol, 2-methoxy-4-(1-propenyl)-	(isoeugenol)	
LIG-6S	Phenol, 2,6-dimethoxy-		
LIG9-G	Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-		
LIG12-S	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-		
LIG13-S	Phenol, 2,6-dimethoxy-4-(2-propenyl)-		
LIG14-G	Phenol, 4-ethyl-2-methoxy-		(Nowakowski and Jones, 2008)
LIG16-S	Phenol, 3,4-dimethoxy-		
LIG17-G	Phenol, 3-methoxy-		
LIG18-G	Phenol, 3-methoxy-2-methyl-		
LIG19-G	Phenol, 2-methoxy-3-methyl-		
LIG22-G	4-Hydroxy-2-methoxybenzaldehyde	(vanillin)	(Nowakowski and Jones, 2008)
LIG26-S	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	(syringaldehyde)	(Ross and Mazza, 2011)
LIG27-G	Homovanillyl alcohol		

Code	Compound	Reference
N1	Indole	(Dignac et *al.*, 2005)
N2	Indole, methyl	
N4	Pyridine	
N5	Pyridine, 3-methyl-	
N6	Pyridine, 2-methyl-	
N8	1H-Pyrrole, 2-methyl-	
LIM	Limonene	
UN1	Toluene	
UN2	Styrene	
UN5	Phenol	
UN6	Phenol, 4-methyl-	
UN7	Phenol, 3-methyl-	
UN8	Phenol, 4-ethyl-	
UN10	1,2-Benzenediol	
UN13	2-Methoxy-4-vinylphenol	(Nowakowski and Jones, 2008
UN14	1,2-Benzenediol, 3-methoxy-	(Ross and Mazza, 2011)
UN15	3-Methoxy-5-methylphenol	

Annex 9. Products identification from untreated wheat straw (UWS) and fungal pretreated straws with several strains (identified by their BRFM number) and their average relative areas per thousand, ranged by increased retention time (RT).

In this table, only an area equal to zero represents the absence of a compound, empty cells only mean a difficulty to find the compound (co-elution, peak too small or absent).

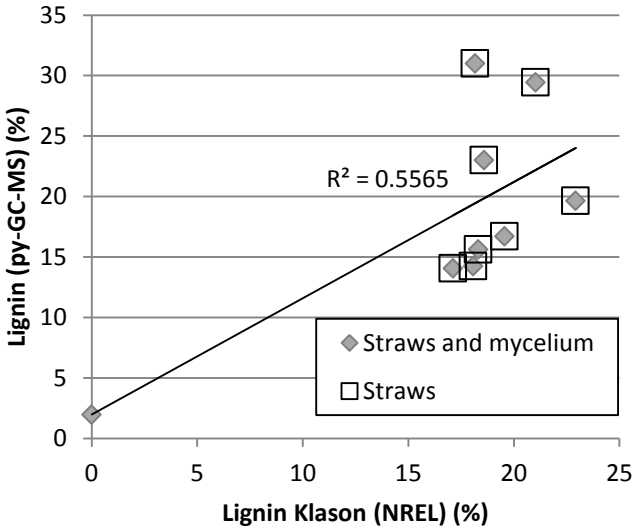
RT	Compound	Code	236	957	985	1048	1296	1554	985-2w	1554-2w	UWS	Mean	SD	SD (% mean)	Empty
6	1,3-Cyclopentadiene	PS46	1.7	15.0	9.0	5.0	11.8	1.8	3.7	0.0	0.0	5.3	5.4	101.3	0
6.2	Furan, 2-methyl-	PS28	6.6			5.2	5.0	23.9	9.3	19.4	9.1	11.2	7.4	66.2	2
7.6	Benzene	61			8.4		24.5			4.7		12.5	10.5	84.2	6
8	1-Heptene	62			2.4							2.4			8
8.5	Furan, 2,5-dimethyl-	PS35	3.1	8.8	4.2	3.3	5.7	6.4	3.0	1.8	5.7	4.7	2.2	46.5	0
9.4	1,3,5-Hexatriene, 2-methyl-	63			1.4							1.4			8
10	Toluene	UN1	5.6	28.9	15.7	11.9	20.4	12.2	12.5	10.8	12.2	14.5	6.7	46.2	0
10.2	Pyridine	N4								4.1		4.1			8
11.4	3-Furaldehyde	PS37		5.4	4.3		6.0					5.2	0.9	16.7	6
12	Furfural	PS25	138.3	41.5		61.2	46.8	129.9	33.1	26.3	37.3	64.3	44.3	68.9	1
12.3	2-Furanmethanol	PS12			1.9							1.9			8
12.5	Ethylbenzene	64	1.1	5.4	4.2	4.0	4.6	2.0				3.6	1.6	46.1	3
12.5	5,9-Dodecadien-2-one, 6,10-dimethyl-, (E,E))-	65			2.1							2.1			8
12.7	Xylene (o or p)	66	3.8	12.0	13.0	13.8	11.2		10.0	10.7	8.4	10.3	3.2	30.5	1
12.9	1-Nonene	67		2.0	2.4							2.2	0.2	11.2	7
13.3	Styrene	UN2		17.7	10.5		9.6	7.7			5.8	10.3	4.5	44.2	4
13.3	1,3,5,7-Cyclooctatetraene	68							6.4	4.6		5.5	1.3	22.9	7
13.4	2-Cyclopentene-1,4-dione	PS38	0.0	5.5	1.0	4.2	2.6		2.1		5.2	2.9	2.1	71.5	2
13.8	2-Cyclopenten-1-one, 2-methyl-	PS3	1.7	3.8	3.9	4.5	3.7	2.3	6.8	6.3	7.2	4.5	2.0	43.8	0
13.9	Ethanone, 1-(2-furanyl)-	PS6	2.5	3.4	3.3	3.5	3.3	2.6	5.3	5.2	4.9	3.8	1.1	28.8	0
14.1	2(5H)-Furanone	PS29			7.4	12.6			15.5			11.8	4.1	34.8	6
14.6	1,2-Cyclopentanedione	PS30	0.0	10.8	22.7	26.5	12.0		24.8	20.3	25.3	17.8	9.3	52.5	1
14.7	Benzene, 2-propenyl-	69		2.2								2.2			8
15	Benzene, 1-ethyl-3-methyl-	70			4.0		2.1					3.0	1.3	43.9	7
15	Benzene, 1-ethyl-2-methyl-	71			2.8							2.8			8
15.2	2-Furancarboxaldehyde, 5-methyl-	PS9	8.6	9.7	8.2	8.6	7.4	16.9	6.5	5.3	6.8	8.7	3.3	38.6	0
15.4	Benzaldehyde	72		11.0			3.9					7.5	5.0	67.1	7

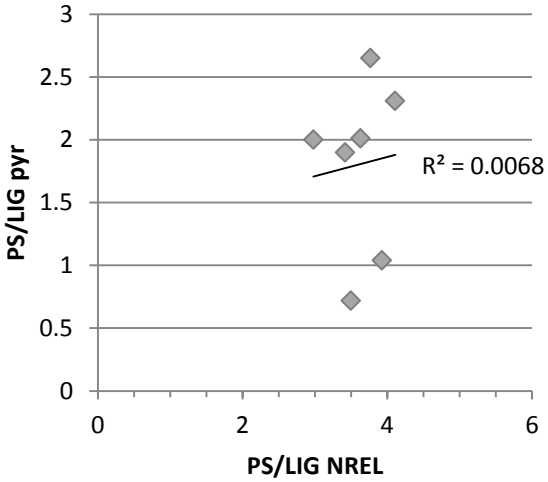
15.5	2-Cyclopenten-1-one, 3-methyl-	PS7	3.8		6.1	10.3		3.6	9.0	9.3	9.1	7.3	2.8	37.8	2
15.5	1-Decene	73		4.0	9.8		2.7					5.5	3.8	68.9	6
15.7	Phenol	UN5	13.2	23.9	17.6	14.2	18.0	22.1	28.8	26.6	25.9	21.1	5.6	26.6	0
15.9	Benzene, 1-methoxy-4-methyl-	74								3.1		3.1			8
15.9	Benzene, 1-ethenyl-3-methyl-	75		3.9	2.6		1.9					2.8	1.0	36.6	6
15.9	Benzene, 1,3,5-trimethyl-	76					4.6			4.2		4.4	0.3	6.7	7
16.1	Benzofuran	77		5.0	2.2		4.5					3.9	1.5	37.9	6
16.7	Limonene	LIM			1.3	2.6	2.5		6.1	6.3	8.4	4.5	2.8	61.0	3
16.9	1,2-Cyclopentanedione, 3-methyl-	PS16	9.2	8.3	12.8	11.6	9.1		28.7	20.5	32.5	16.6	9.5	57.3	1
17	2-Cyclopenten-1-one, 2,3-dimethyl-	PS8		6.2	3.9	4.0	4.0		8.0	6.9	6.8	5.7	1.7	29.7	2
17.1	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	PS34						6.2				6.2			8
17.4	Indene	78			3.1	3.0	3.8	2.4		4.0		3.3	0.6	19.5	4
17.5	Phenol, 4-methyl-	UN6			23.5	16.8	19.3	30.5		28.7		23.8	5.9	24.7	4
17.5	Phenol, 2-methyl-	79			15.0	11.2	12.1		18.1	15.3	17.0	14.8	2.7	18.2	3
17.9	Phenol, 3-methyl-	UN7	14.8	32.6					34.1		34.0	28.9	9.4	32.5	5
18.3	Phenol, 2-methoxy-	LIG1-G	24.6	24.5	19.4	20.8	11.1	21.2	51.4	40.0	63.5	30.7	17.2	56.0	0
18.8	Benzofuran, 2-methyl-	PS42	3.6	7.1	6.3		6.1	11.2		4.5		6.5	2.6	41.0	3
19	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	PS31									9.2	9.2			8
19	1,3-Cyclopentanedione, 2,2-dimethyl-	PS33							10.5			10.5			8
19	Maltol	PS36			6.4							6.4			8
19.1	Levoglucosenone	PS18	0.0	8.4	5.3	15.0	13.5		5.8	PS31	3.5	7.4	5.4	72.9	1
19.3	Phenol, 2-ethyl-	80		2.6	1.5		2.8					2.3	0.7	31.3	6
19.6	Phenol, 2,4-dimethyl-	81		9.3	9.0	5.9	8.8	2.9		13.1		8.2	3.5	42.3	3
19.6	Phenol, 2,5-dimethyl-	82	7.3			3.9	3.9	9.2				6.1	2.6	43.7	5
19.7	1H-Indene, 1-methyl-	83		4.1	4.4					3.7		4.0	0.3	8.4	6
20	Phenol, 4-ethyl-	UN8	5.3	7.5	7.9	5.4	4.7			9.6	11.1	7.4	2.4	32.9	2
20.4	Phenol, 3-methoxy-2-methyl-	LIG18-G								7.1		7.1			8
20.6	Phenol, 2-methoxy-4-methyl-	LIG2-G	15.2	8.2	8.1	5.2	6.0	12.3	14.0	18.4	19.3	11.9	5.3	44.4	0
20.6	Phenol, 3,5-dimethyl-	85							9.7		22.6	16.2	9.1	56.5	7
20.7	1,2-Benzenediol	UN10	48.6		36.1				31.3	32.5	16.9	33.1	11.3	34.3	4
20.8	Azulene	86					3.8					3.8			8
21.2	Benzofuran, 2,3-dihydro-	87				11.6		7.9	13.3	8.6	18.2	11.9	4.1	34.7	4
21.5	Phenol, 3-methoxy-	LIG17-G								2.2		2.2			8

21.5	1,4:3,6-Dianhydro-.alpha.-d-glucopyranose	PS21	16.8	14.5	13.1	14.0	5.9		9.2	6.0	12.3	11.5	4.0	34.9	1
21.7	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	PS26			6.7	8.6	8.7					8.0	1.1	13.9	6
21.7	Anhydro-d-mannosan	PS43'		8.3								8.3			8
22.2	1,2-Benzenediol, 3-methoxy-	UN14						23.8	37.0	29.0	18.6	27.1	7.8	28.9	5
22.3	Hydroquinone	88								10.3		10.3			8
22.4	Phenol, 4-ethyl-2-methoxy-	LIG14-G		10.7	9.3	7.5	11.1		12.5		18.8	11.6	3.9	33.5	3
22.7	4-Hydroxy-2,4,5-trimethyl-2,5-cyclohexadien-1-one	89	7.5									7.5			8
22.8	Bicyclo[4.2.0]octa-1,3,5-triene	90			6.0							6.0			8
22.9	1,2-Benzenediol, 4-methyl-	91	20.9							11.9	10.9	14.6	5.5	38.0	6
23	5-Acetoxymethyl-2-furaldehyde	PS45		2.1								2.1			8
23	Naphthalene, 1-methyl-	92		4.3								4.3			8
23.1	Naphthalene, 2-methyl-	93					7.9			3.0		5.4	3.5	64.2	7
23.1	Benzocycloheptatriene	95			3.3			1.8				2.6	1.1	41.8	7
23.4	3-Methoxy-5-methylphenol	UN15		4.3								4.3			8
23.4	2-Methoxy-4-vinylphenol	UN13	19.1	5.6	8.7	10.9	11.3		31.0	21.0	61.8	21.2	18.3	86.6	1
23.6	Phthalic anhydride	96					6.7					6.7			8
24	Phenol, 2,6-dimethoxy-	LIG6-S	21.6	13.6	16.9	12.3	13.6	23.5	49.4	42.0	60.1	28.1	17.8	63.3	0
24.2	Tetradecane	97		2.3	1.6		5.6					3.2	2.1	66.7	6
24.2	1-Tetradecene	98		5.4								5.4			8
24.3	Phenol, 3,4-dimethoxy-	LIG16-S								7.0		7.0			8
24.4	N-Benzyl-2-[1-(4-methoxy-phenyl)-1H-tetrazol-5-ylsulfanyl]-acetamide	99					3.0					3.0			8
24.6	1,3-Benzenediol, 4-ethyl-	100								8.1	11.4	9.8	2.4	24.2	7
24.7	Phenol, 2-methoxy-3-methyl-	LIG19-G	3.5									3.5			8
25	Eugenol	LIG4-G							3.6	5.6	9.3	6.2	2.9	46.8	6
25.1	Vanillin	LIG22-G	11.1	4.7	7.8	8.7	8.9	7.6	8.7	8.9	9.1	7.9	2.5	31.5	0
25.4	2-Methyl-5-hydroxybenzofuran	PS40					5.8	3.1				4.4	1.9	43.9	7
25.7	4-Methoxy-2-methyl-1-(methylthio)benzene	101		6.9					14.4			10.7	5.3	49.7	7
25.7	2H-Pyran-2-one, 3-acetyl-4-hydroxy-6-methyl-	102					7.2				15.5	11.3	5.9	51.7	7

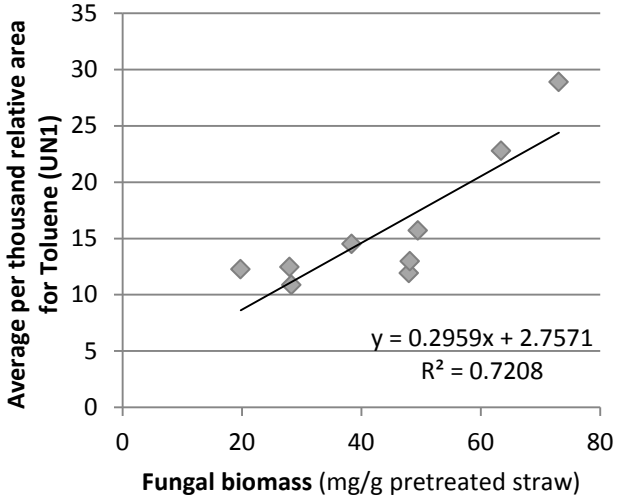
26	Phenol, 2-methoxy-4-(1-propenyl)-	LIG5-G	9.0		4.6		5.0		12.6	12.4	16.8	10.1	4.8	47.3	3
26	Pentadecane	103		3.0								3.0			8
26.1	1-Pentadecene	104			3.5							3.5			8
26.7	Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-	LIG9-G	5.4	5.4		4.2	6.6		8.4			6.0	1.6	26.1	4
27.1	Benzoic acid, 4-hydroxy-3-methoxy-, methyl ester	105				1.9	3.2					2.6	0.9	34.6	7
27.4	Homovanillyl alcohol	UN11									5.7	5.7			8
27.5	1,6-Anhydro-.beta.-D-glucopyranose (levoglucosan)	PS24		8.2		47.1						27.6	27.5	99.6	7
27.6	4(5H)-Benzofuranone, 6,7-dihydro-3,6-dimethyl-, (R)-		7.3						5.1			6.2	1.5	24.6	7
27.7	D-Allose	PS44		45.5	58.4							51.9	9.1	17.6	7
28.4	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	LIG13-S	4.6		2.6	6.1	5.7	16.6	7.5	10.1	12.9	8.3	4.7	56.2	1
28.6	Fluorene	106		3.9								3.9			8
29.5	1-Heptadecene	107			1.9		2.0					1.9	0.1	4.9	7
29.7	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	LIG26-S	2.3	2.5	1.1	2.9	4	2.0				2.1	0.7	35.9	5
30	Cyclohexane, 2-butyl-1,1,3-trimethyl-	108			2.0							2.0			8
30.8	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	LIG12-S	4.7		4.5	3.6	6.9	4.7	7.7	4.9	7.8	5.6	1.6	28.5	1

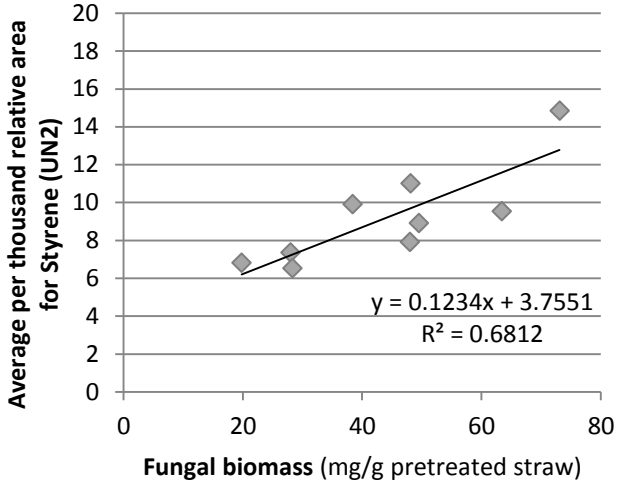
Annex 10. Relationship between pyrolysis-GC-MS data and NREL (acid hydrolysis) measurements for straws from Table 5-2.





Annex 11. Correlations between fungal biomass determined by qPCR and Toluene (UN1) and Styrene (UN2) relative areas in pyrograms of fungal pretreated wheat straws.

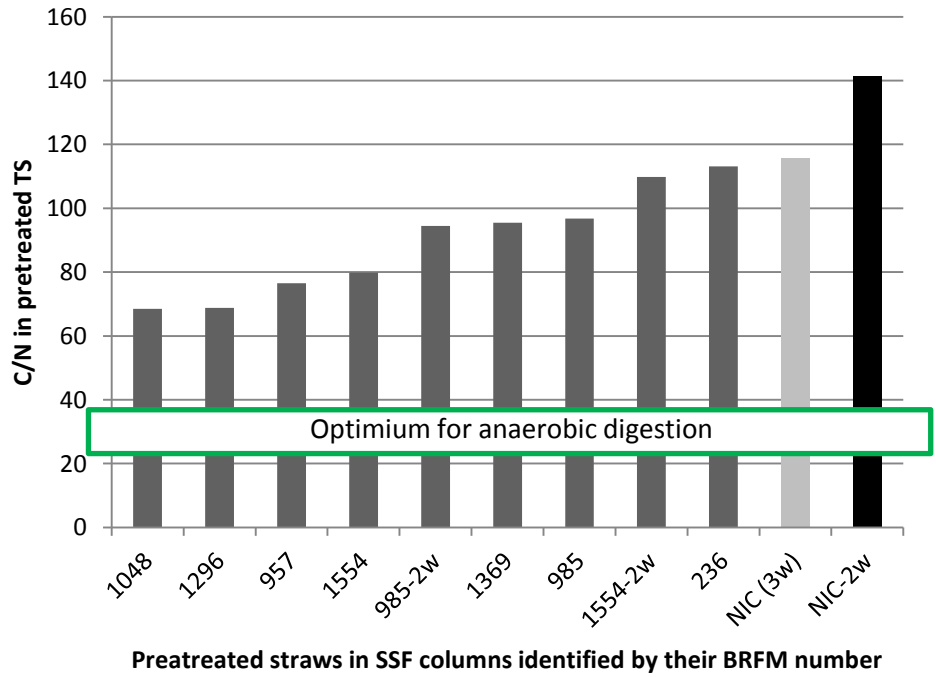




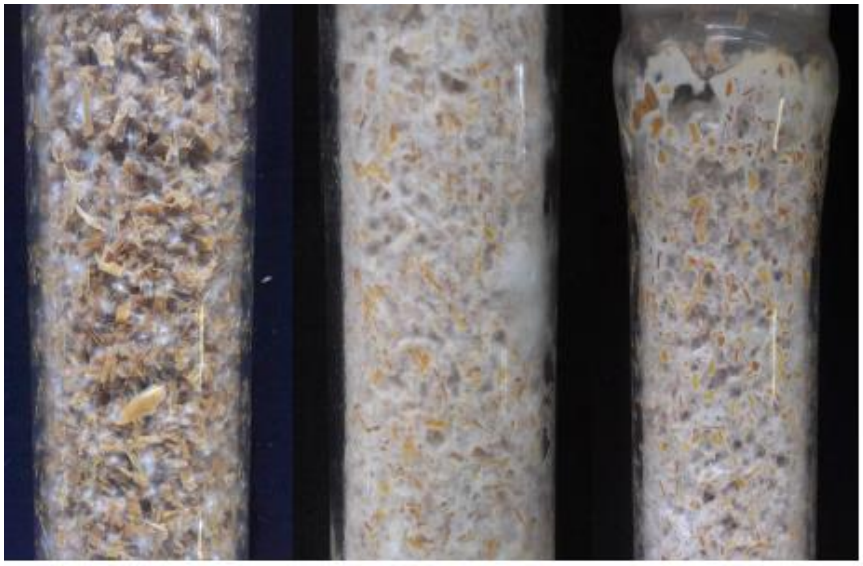
Annex 12. *P. brumalis* BRFM 985 growth in pilot-reactor on wheat straw (two trays with different particle sizes) whitout fungal contamination.

Annex 13. C/N of pretreated straws during the selection step.

w:weeks



Annex 14. Homogeneous fungal growth in glass columns.
From S. Zhou.



Annex 15. Fungal contamination after trial of wheat straw pretreatment with *P. brumalis* BRFM 985 in the LBE pilot-reactor.

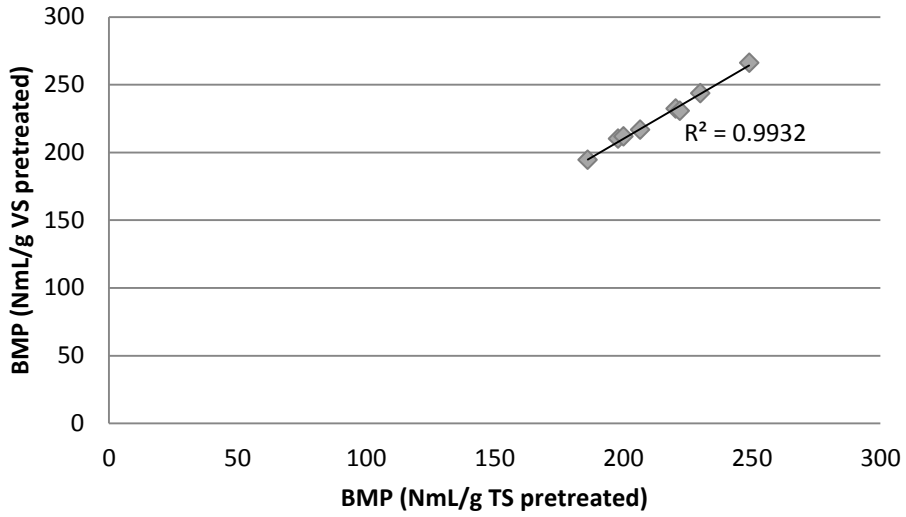
Annex 16. Successfull growth of *P. brumalis* BRFM 985 on miscanthus straw pretreated in pilot-reactor.

Annex 17. Data used to plot the PCA in Chapter 7.

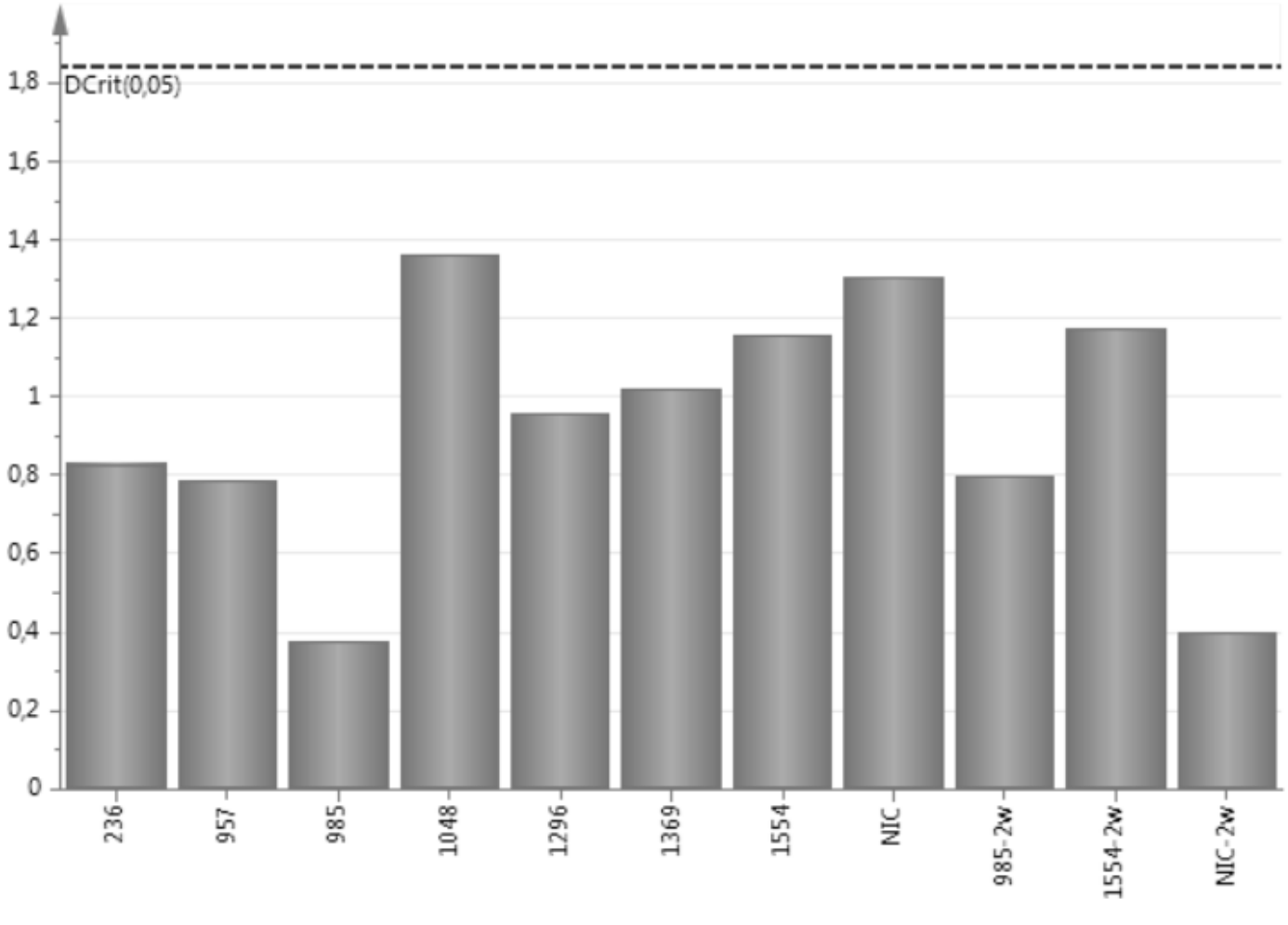
As mentioned in chapter 7, units are in %TS for non dimensionless variables and BMP is expressed in NmL/g VS pretreated. N. A.: Not Available.

	TC	TKN	PS/LIG -pyr	PS/LIG NREL	S/G -pyr	PS1/ LIG	PS2/ LIG	BMP	Fungi	Yield	Cellulose	Hemi	Lignin	Cel Loss	Hemi Loss	LIG Loss	LOI
236	46.31	0.41	1.84	2.98	0.49	1.81	1.75	216.64		92.27	39.39	28.90	22.93	10.49	18.92	7.83	1.10
957	43.91	0.57	2.74	3.77	0.36	2.18	2.06	266.10	73.10	71.22	38.30	29.87	18.08	39.90	43.98	51.40	1.10
985	44.93	0.53	2.11	4.11	0.38	2.09	1.93	279.52	49.50	86.34	41.05	29.34	17.12	16.10	28.34	40.10	1.11
1048	43.81	0.64	N. A.	3.81		1.96	1.87	232.22	48.00	85.13	38.82	30.90	18.31	17.60	21.62	35.20	1.06
1296	44.26	0.64	2.01	3.42	0.52	2.16	2.06	243.43		84.37	36.40	30.48	19.56	24.94	25.18	30.92	1.05
1369	44.35	0.46	N. A.	3.54		2.08	2.02	209.96	63.50	79.28	37.47	30.73	19.29	28.10	37.30	29.63	1.04
1554	44.34	0.56	0.69	3.62	0.44	2.08	2.03	211.57	19.80	84.79	36.77	30.63	18.59	24.80	25.17	34.40	1.08
NIC	46.01	0.40	1.57	3.15	0.40	N. A.	N. A.	194.42	0.00	100.00	37.48	31.45	21.90	0.00	0.00	0.00	N. A.
985-2w	45.31	0.48	0.83	3.93	0.57	2.17	1.98	_	28.00	87.40	39.45	31.95	18.16	15.50	5.24	24.04	1.04
1554-2w	45.85	0.48	0.69	3.50	0.44	2.15	2.02	_	28.30	85.60	41.49	32.01	21.03	12.95	5.28	13.87	1.06
NIC-2w	47.11	0.33	1.84	3.35	N. A.	1.81	1.64	_	0.00	100.00	40.80	29.30	20.90	0.00	0.00	0.00	1.16

Annex 18. Correlation between BMP values in NmL/g VS pretreated and BMP values in NmL/g TS pretreated from samples from the selection step.



Annex 19. DmodX values in the space defined by three components for samples from the PCA presented in Chapter 7.



FUNGAL PRETREATMENTS FOR LIGNOCELLULOSIC BIOMASS ANAEROBIC DIGESTION

Anaerobic digestion of lignocellulosic biomass is the most efficient way to produce renewable energy. However, lignin contained in this biomass is difficult to hydrolyze. This limitation can be overcome by pretreatments. Among them, white-rot fungi (WRF) pretreatments seem attractive but were scarcely applied for anaerobic digestion. The current study investigates WRF pretreatments of wheat straw to improve its methane production. Firstly, a selection step has revealed the efficiency of Polyporus brumalis BRFM 985 since 43% more methane per gram of pretreated volatile solids were obtained compared to the control straw. Taking into account the dry weight loss occurring during the pretreatment, it still corresponded to 21% more methane per gram of initial total solids. Moreover, glucose addition during the pretreatment was shown to limit delignification and thus methane production from the substrate. Secondly, an experiment device aiming to optimize the pretreatment with P. brumalis BRFM 985 was carried out; tested pretreatments parameters were: culture duration, temperature, initial substrate moisture content and metals addition. Response surfaces of methane production from those samples were built. Optimum methane production was not reached in the experimental domain but the positive impact of metals addition was demonstrated, so as the importance to choose adequate culture duration. Then, the use of pyrolysis-GC-MS technic to access pretreatment efficiency was studied. Estimation of fungal biomass amount on wheat straw with this method appeared possible. Polysaccharides/lignin ratio determined with py-GC-MS allowed to classify some pretreated samples according to their anaerobic degradability. Solid State Anaerobic Digestion (SSAD) of wheat straw pretreated in pilot-reactor was carried out in batch with leachate recycle. With wheat straw, Substrate/Inoculum (S/I) between 2 and 3 (Volatile Solid basis) allow a successful start-up in SSAD. Whereas Total VFA/alkalinity ratio under 0.6 corresponds to stable wet anaerobic digestion; this limit seems not well adapted to SSAD. It was observed that SSAD reactors were able to recover from acidification phase when Total VFA/alkalinity was lower than 2 and with VFA concentrations inferior to 10 g/L in leachate. Despite the improvement of biodegradability and the facilitation of start-up phase, non-optimized fungal pretreatment did not improve methane production after taking into account mass losses occurring during the pretreatment.

DEFENDED ON 17TH DECEMBER 2015 AT : Lbe

WITH THE FINANCIAL SUPPORT OF : ANR

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