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# Chemical and fungal pretreatments of lignocellulosic biomass for biogas production

Shruthi Meenakshisundaram

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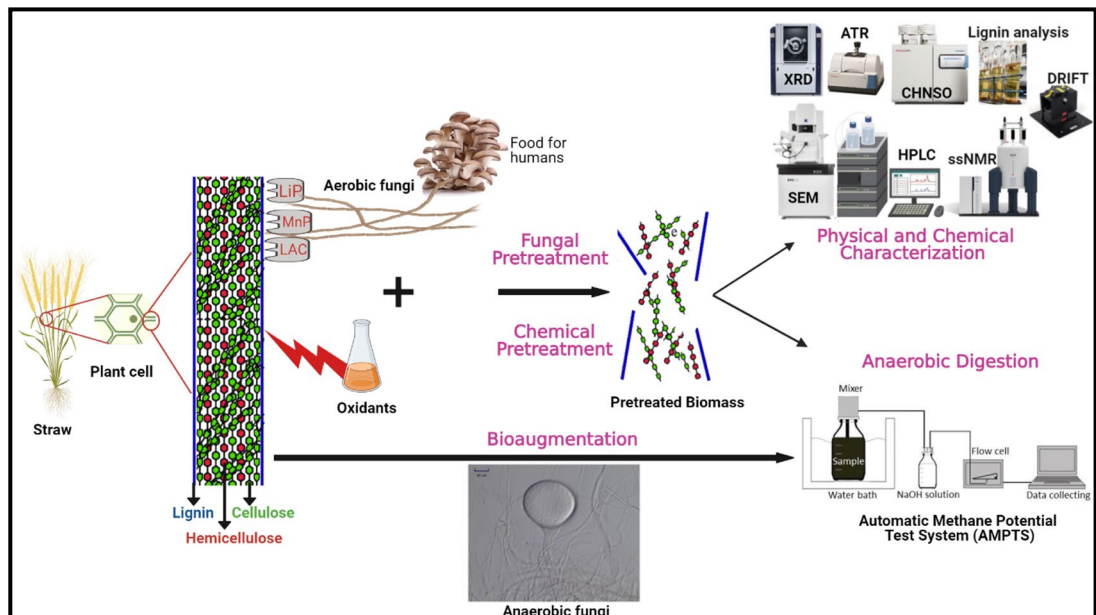
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Par **Shruthi MEENAKSHISUNDARAM**

*Chemical and fungal pretreatments of lignocellulosic biomass for biogas production*

Thèse présentée  
pour l'obtention du grade  
de Docteur de l'UTC



Soutenue le 20 mars 2023

**Spécialité :** Génie des Procédés et Bioprocédés : Transformations intégrées de la matière renouvelable (EA-4297)

D2726



# Chemical and fungal pretreatments of lignocellulosic biomass for biogas production

Thèse présentée et soutenue à Compiègne, le 20 mars 2023

par **Shruthi Meenakshisundaram**

pour obtenir le grade de docteur de l'Université de Technologie de Compiègne

École doctorale ED 71 UTC Sciences pour l'ingénieur

**Spécialité : Génie des Procédés et Bioprocédés**

Transformations Intégrées de la Matière Renouvelable (TIMR, UTC-ESCOM)

Devant le jury composé de :

Craig FAULDS, Professeur, INRAE-Université d'Aix-Marseille	Rapporteur
Thierry RIBEIRO, Enseignant-chercheur, Institut Polytechnique	Rapporteur
UniLaSalle Claude JOLIVALT, Professeur, Sorbonne Université	Examinatrice
Sabine PODMIRSEG, Assistant Professeur, University of Innsbruck	Examinatrice
Erwann GUÉNIN, Professeur, UTC	Examinateur
Claire CEBALLOS, Enseignant-chercheur, ESCOM	Directrice de thèse
André PAUSS, Professeur, UTC	Directeur de thèse
Antoine FAYEULLE, Maître de conférences, UTC	Co-encadrant
Estelle LÉONARD, Enseignant-chercheur, ESCOM	Invitée
Xiaojun LIU, Maître de conférences, UTC	Invité
Vincenzo CALCAGNO, Post-doctorant, Sorbonne Université	Invité



## **DEDICATION**

To the woman who raised me,  
my paternal grandmother, **SEETHALAKSHMI**,  
who's been a rock of stability throughout my life  
and  
my father, **MEENAKSHISUNDARAM**,  
without whom none of my success would be possible



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## Liste des publications et communications

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chemical treatments. In: 3<sup>rd</sup> International Conference for Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, 17-19 May 2021.

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Meenakshisundaram S., Fayeulle A., Leonard E., Ceballos C. and Pauss A. (2021). Improvement of methanization yields of lignocellulosic biomass by coupling fungal and chemical treatments. In: Journée des Jeunes Chercheurs 2021 UGéPE-GEPROC, 25<sup>th</sup> November 2021, Dunkerque, France.

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Meenakshisundaram S., Fayeulle A., Leonard E., Ceballos C., Liu X. and Pauss A. (2022). Improvement of anaerobic digestion of lignocellulosic biomass by coupling fungal and chemical treatments. In: 4<sup>th</sup> International Conference on Biogas Microbiology, 9-11 May 2022, Braga, Portugal.

## Abstract

Two challenges facing mankind currently are reducing and/or treating waste and decreasing the dependency on fossil fuel resources. The transformation of organic waste into energy by developing integrated biorefineries will help to solve the two problems while creating a circular economy. Biogas production from organic waste can provide a renewable and reliable source of energy and has the advantage of being able to upgrade to transport fuel, cooking gas, or electricity. Lignocellulosic biomass is an abundant organic waste and a cheap resource for energy generation. However, the physical and chemical rigidity of the feedstock makes it a difficult and expensive process to convert biomass into biofuels. A pretreatment step helps to reduce the recalcitrance and prepares the substrate for the downstream process. Each pretreatment method has its advantages and disadvantages and therefore, combining two pretreatment steps helps to lower the severity of drawbacks of a single pretreatment step.

In this thesis, the influence of both combined physical-chemical pretreatment and combined biological-chemical pretreatment on biogas production from straw was studied. In the case of physical-chemical pretreatment, the physical pretreatment was observed to mask the synergy of combined pretreatment for enhancing biogas production. However, even with a wide range of analytical techniques, the structural changes in the biomass caused by physical and chemical pretreatment are difficult to explain. Therefore, the rationale behind the impact of physically and chemically pretreated biomass on biogas production is hard to understand.

Two spent mushroom substrate which is a pre-degraded biomass waste generated during industrial mushroom cultivation was studied for biogas production. It was observed that though both the strains studied had the same lineage and are commercially used for mushroom production under solid-state cultivation, they do not degrade the biomass in the same way. Consequently, they show a difference in the biomethane yield. Therefore, when a strain for mushroom production is chosen, it needs to be studied for its biogas production capability to develop an integrated “waste-to-energy” industry. Similar variations in the biomass-degrading capacity in submerged conditions were detected among three pure strains of *Pleurotus ostreatus* obtained from the mycology bank. The study was conducted in submerged conditions to have a more homogenous environment for fungal pretreatment. As a result of these two studies on fungal pretreatment, the ability of the same strain to alter the biomass differently based on the culture condition (solid-state and submerged conditions) was observed. The efficiency of fungal pretreatment is dependent on the strain used and the culture conditions. Thus, a systematic and

thorough selection of appropriate fungal isolates is needed for selecting the best strain for an effective deconstruction of lignocellulosic biomass.

The combined biological-chemical pretreatment was carried out with minimal size reduction and no autoclave process, in order to be a less energy-intensive process. Again, the effect of combined pretreatment was particular to each strain of fungi used. Of the two strains compared, sole fungal pretreatment with PO3 was sufficient enough to increase the biogas production while the combined pretreatment was needed for HK35 to considerably increase biogas production. The order of pretreatment was also seen to influence biogas production. In the case of chemical pretreatment followed by fungal pretreatment, the chemical pretreatment made more of the polysaccharides available for fungal growth, which reduced the amount of available carbohydrates to be converted into biomethane. The fungal pretreatment followed by chemical pretreatment was seen to be beneficial for the HK35 strain but not for PO3. This is probably because the HK35 strain did not use up so much of the inaccessible polysaccharides for its growth which could have been released by additional Fenton pretreatment. Therefore, the two strains of the same species are seen to have different modes of action that impact biomass characteristics and biogas production differently. Thus, the severity of physical/chemical pretreatment, the fungal species used, the culture conditions, and the order of sequential pretreatment are all observed to influence the relative components of biomass and biogas production.

An alternative to pretreatment strategies is bioaugmentation, in which separately grown microbes with specific activity for a metabolic pathway are added to a digester to improve the efficiency of the microbial community in biogas production. Anaerobic fungi have cellulolytic machinery that produces a plethora of hydrolyzing enzymes and unlike aerobic fungi, there is no loss of plant carbohydrates due to respiration. The synergistic use of mechanical and enzymatic degradation makes anaerobic fungi a promising approach to improve biogas production. In this study, bioaugmentation with a considerably low quantity of *Feramyces austinii* was seen to improve the biomethane yield slightly. This has opened up new possibilities to exploit the enzyme systems and metabolism of anaerobic fungi for lignocellulosic biomass degradation.

**Keywords: Lignocellulosic biomass, Anaerobic digestion, Combined pretreatment, Fenton pretreatment, *Pleurotus ostreatus*, Bioaugmentation, Anaerobic fungi**

## Résumé

La réduction et/ou le traitement des déchets et la diminution de la dépendance vis-à-vis des ressources en combustibles fossiles sont deux défis auxquels l'humanité est actuellement confrontée. La transformation des déchets organiques en énergie par le développement de bioraffineries intégrées contribuera à résoudre ces deux problèmes tout en créant une économie circulaire. La production de biogaz à partir de déchets organiques peut constituer une source d'énergie renouvelable et fiable et présente l'avantage de pouvoir être transformée en carburant pour le transport, en gaz de cuisson ou en électricité. La biomasse lignocellulosique est un déchet organique abondant et une ressource bon marché pour la production d'énergie. Cependant, la rigidité physique et chimique de la matière première rend le processus de conversion de la biomasse en biocarburants difficile et coûteux. Une étape de prétraitement permet de réduire la récalcitrance et de préparer le substrat pour le processus en aval. Chaque méthode de prétraitement a ses avantages et ses inconvénients et, par conséquent, la combinaison de deux étapes de prétraitement permet de réduire la gravité des inconvénients d'une seule étape de prétraitement.

Dans cette thèse, l'influence du prétraitement physico-chimique combiné et du prétraitement biologique-chimique combiné sur la production de biogaz à partir de la paille a été étudiée. Dans le cas du prétraitement physico-chimique, il a été observé que le prétraitement physique masquait la synergie du prétraitement combiné pour l'amélioration de la production de biogaz. Cependant, même avec un large éventail de techniques analytiques, les changements structurels de la biomasse provoqués par les prétraitements physiques et chimiques sont difficiles à expliquer. Par conséquent, il est difficile de comprendre la logique qui sous-tend l'impact de la biomasse prétraitée physiquement et chimiquement sur la production de biogaz.

Deux substrats de champignons usés, qui sont des déchets de biomasse pré-dégradés générés lors de la culture industrielle de champignons, ont été étudiés pour la production de biogaz. Il a été observé que, bien que les deux souches étudiées aient la même lignée et soient utilisées commercialement pour la production de champignons en culture en milieu solide, elles ne dégradent pas la biomasse de la même manière. Par conséquent, elles présentent une différence dans le rendement en biométhane. Par conséquent, lorsqu'une souche pour la production de champignons est choisie, elle doit être étudiée pour sa capacité de production de biogaz afin de développer une industrie intégrée "des déchets à l'énergie". Des variations

similaires de la capacité de dégradation de la biomasse dans des conditions d'immersion ont été détectées parmi trois souches pures de *Pleurotus ostreatus* obtenues à partir de la banque de mycologie. L'étude a été menée dans des conditions d'immersion afin d'obtenir un environnement plus homogène pour le prétraitement fongique. Ces deux études sur le prétraitement fongique ont permis d'observer la capacité d'une même souche à modifier la biomasse différemment en fonction des conditions de culture (à l'état solide et en conditions immergées). L'efficacité du prétraitement fongique dépend de la souche utilisée et des conditions de culture. Ainsi, une sélection systématique et approfondie des isolats fongiques appropriés est nécessaire pour sélectionner la meilleure souche pour une déconstruction efficace de la biomasse lignocellulosique.

Le prétraitement biologique-chimique combiné a été effectué avec une réduction minimale de la taille et sans autoclave, afin d'être un processus moins énergivore. Une fois encore, l'effet du prétraitement combiné était propre à chaque souche de champignon utilisée. Sur les deux souches comparées, le seul prétraitement fongique avec PO3 était suffisant pour augmenter la production de biogaz, tandis que le prétraitement combiné était nécessaire pour HK35 afin d'augmenter considérablement la production de biogaz. L'ordre de prétraitement a également influencé la production de biogaz. Dans le cas d'un prétraitement chimique suivi d'un prétraitement fongique, le prétraitement chimique a rendu davantage de polysaccharides disponibles pour la croissance fongique, ce qui a réduit la quantité d'hydrates de carbone disponibles à convertir en biométhane. Le prétraitement fongique suivi d'un prétraitement chimique s'est avéré bénéfique pour la souche HK35, mais pas pour PO3. Ceci est probablement dû au fait que la souche HK35 n'a pas utilisé pour sa croissance une grande quantité de polysaccharides inaccessibles qui auraient pu être libérés par un prétraitement Fenton supplémentaire. Par conséquent, les deux souches de la même espèce semblent avoir des modes d'action différents qui ont un impact différent sur les caractéristiques de la biomasse et la production de biogaz. Ainsi, la sévérité du prétraitement physique/chimique, l'espèce fongique utilisée, les conditions de culture et l'ordre du prétraitement séquentiel influencent les composantes relatives de la biomasse et de la production de biogaz.

Une alternative aux stratégies de prétraitement est la bioaugmentation, dans laquelle des microbes cultivés séparément et ayant une activité spécifique pour une voie métabolique sont ajoutés à un digesteur pour améliorer l'efficacité de la communauté microbienne dans la production de biogaz. Les champignons anaérobies possèdent une machinerie cellulolytique

qui produit une pléthore d'enzymes d'hydrolyse et, contrairement aux champignons aérobies, il n'y a pas de perte d'hydrates de carbone végétaux due à la respiration. L'utilisation synergique de la dégradation mécanique et enzymatique fait des champignons anaérobies une approche prometteuse pour améliorer la production de biogaz. Dans cette étude, la bioaugmentation avec une quantité considérablement faible de *Feromyces austinii* a permis d'améliorer légèrement le rendement en biométhane. Cela a ouvert de nouvelles possibilités d'exploitation des systèmes enzymatiques et du métabolisme des champignons anaérobies pour la dégradation de la biomasse lignocellulosique.

**Mot clés : Biomasse lignocellulosique, Digestion anaérobie, Prétraitement combiné, Prétraitement Fenton, *Pleurotus ostreatus*, Bioaugmentation, Champignons anaérobies**





# Table of contents

Acknowledgments .....	i
Liste des publications et communications .....	iii
Abstract .....	v
Résumé .....	vii
List of abbreviations .....	xv
I General Introduction .....	1
II Research Objectives and scientific approach .....	7
III Literature Review .....	11
<b>III.1 Fiber degradation and carbohydrate production by combined biological and chemical/ physicochemical pretreatment methods of lignocellulosic biomass – A review.</b> .....	<b>11</b>
III.1.1 Introduction.....	11
III.1.2 Use of fiber content degradation analysis to evaluate the combined pretreatment effect .....	14
III.1.3 Evaluation of the combined pretreatment based on sugar yield.....	33
III.1.4 Conclusion.....	59
<b>III.2 Combined Biological and Chemical/Physicochemical Pretreatment Methods of Lignocellulosic Biomass for Bioethanol and Biomethane Energy Production – A Review</b> .....	<b>61</b>
III.2.1 Introduction.....	61
III.2.2 Comparison of combined pretreatment based on biogas yields .....	66
III.2.3 Comparison of combined pretreatment based on bioethanol yields.....	71
III.2.4 Properties of the employed microorganisms in the pretreatment step.....	81
III.2.5 New trends in microorganisms used in downstream process .....	84
III.2.6 Challenges and possible solutions .....	86
III.2.7 Conclusion.....	87
<b>III.3 Lignin Fungal Depolymerization: From Substrate Characterization to Oligomers Valorization</b> .....	<b>89</b>
III.3.1 Introduction.....	90
III.3.2 Analytical Methods Adapted to Lignin Characteristics.....	91
III.3.3 Fungal Enzymes and Mechanisms Involved During Lignin Depolymerization .....	108
III.3.4 Conclusion and Future Perspectives.....	121
<b>III.4 A perspective summary of Chapter III.....</b>	<b>122</b>

<b>IV Chemical Pretreatment of Lignocellulosic Biomass .....</b>	<b>125</b>
<b>IV.1 Optimization of oxidant concentration .....</b>	<b>125</b>
IV.1.1 Introduction .....	125
IV.1.2 Materials and Methods .....	127
IV.1.3 Results and Discussion .....	129
IV.1.4 Conclusion and Perspectives.....	134
<b>IV.2 Chemically and physically pretreated straw in moderate conditions: Poor correlation between biogas production and commonly used biomass characterization .....</b>	<b>135</b>
IV.2.1 Introduction .....	135
IV.2.2 Materials and Methods .....	138
IV.2.3 Results.....	145
IV.2.4 Discussion and Perspectives .....	162
IV.2.5 Conclusions .....	165
<b>IV.3 A perspective summary of Chapter IV .....</b>	<b>165</b>
<b>V Fungal pretreatment of lignocellulosic biomass .....</b>	<b>167</b>
<b>V.1 Utilization of spent mushroom substrate for biogas production .....</b>	<b>167</b>
V.1.1 Introduction .....	167
V.1.2 Materials and Methods .....	168
V.1.3 Results and Discussion .....	169
V.1.4 Conclusion.....	178
<b>V.2 Degradation of straw by three different strains of Pleurotus ostreatus.....</b>	<b>181</b>
V.2.1 Introduction .....	181
V.2.2 Materials and Methods .....	181
V.2.3 Results and Discussion .....	184
V.2.4 Conclusion.....	188
<b>V.3 A perspective summary of Chapter V .....</b>	<b>188</b>
<b>VI Combined fungal and chemical pretreatment of lignocellulosic biomass .....</b>	<b>189</b>
<b>VI.1 Introduction .....</b>	<b>189</b>
<b>VI.2 Materials and Methods .....</b>	<b>189</b>
VI.2.1 Materials .....	189
VI.2.2 Pretreatment Strategies.....	190
VI.2.3 Biomass Characterization.....	191
VI.2.4 Biomethane Potential Test .....	191
<b>VI.3 Results and Discussion.....</b>	<b>191</b>

VI.3.1 Biomass Characterization.....	191
VI.3.2 Lignin Composition .....	192
VI.3.3 Crystallinity index and crystallite size using WAXD.....	193
VI.3.4 Scanning Electron Microscopy Analysis.....	194
VI.3.5 Biomethane Potential Test .....	197
<b>VI.4 Conclusion and a perspective summary of Chapter VI .....</b>	<b>199</b>
<b>VII Perspectives of usage of anaerobic fungi for biogas production .....</b>	<b>201</b>
<b>VII.1 Introduction .....</b>	<b>201</b>
<b>VII.2 Materials and Methods .....</b>	<b>201</b>
VII.2.1 Strain Maintenance .....	201
VII.2.2 Bioaugmentation Set-up.....	202
VII.2.3 Lignin Composition .....	203
<b>VII.3 Result and Discussion .....</b>	<b>204</b>
VII.3.1 Strain Morphology .....	204
VII.3.2 Biomethane Potential Test.....	205
VII.3.3 Lignin Composition .....	207
<b>VII.4 Conclusion and a perspective summary of Chapter VII .....</b>	<b>208</b>
<b>VIII General Conclusions and Future Scope .....</b>	<b>209</b>
<b>IX Summary in French.....</b>	<b>213</b>
<b>IX.1 Introduction Générale .....</b>	<b>213</b>
<b>IX.2 Les objectifs de recherche et approche scientifique .....</b>	<b>219</b>
<b>IX.3 Conclusions générales et perspectives .....</b>	<b>222</b>
<b>X References .....</b>	<b>227</b>
<b>XI Annex.....</b>	<b>259</b>
<b>XI.1 Chemically and physically pretreated straw in moderate conditions: Poor correlation between biogas production and commonly used biomass characterization – Supplementary Materials .....</b>	<b>259</b>



## List of abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AD	Anaerobic digestion
ADF	Acid detergent fiber
ADL	Acid detergent lignin
AF	Anaerobic fungi
AFEX	Ammonia fiber explosion
AFM	Atomic force microscopy
AIL	Acid insoluble lignin
AMPTS	Automatic methane potential test system
ANOVA	Analysis of Variance
AOAC	Association of official analytical chemists
ASL	Acid soluble lignin
ATP	Adenosine triphosphate
BMP	Biomethane potential
BRF	Brown rot fungi
C/N	Carbon to nitrogen ratio
CI	Crystallinity index
CLL	Cross-linked lignin
CP	Chemical pretreatment
CP-MAS	Cross-polarization magic-angle-spinning
DB	Direct blue dye
DFRC	Derivatization followed by reductive cleavage
DMC	Direct microbial conversion
DNS	3,5-dinitrosalicylic acid

DO	Direct orange dye
DRIFT	Diffuse reflectance infrared Fourier transform spectroscopy
DSC	Differential enthalpy analysis
DW	Demineralized water
DyP	Dye decolourizing peroxidases
EDS	Energy dispersive spectroscopy
ELSD	Evaporative light scattering detector
FE-SEM	Field emission – Scanning electron microscopy
FOS/TAC	Fluchtige Organische Sauren/ Total Anorganic Carbon
FP	Fungal pretreatment
fPoXDB	Fungal peroxidases database
FTIR	Fourier-transform infrared spectroscopy
FWMH	The full width at half maximum
G	Glucose
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HAA	3-hydroxyantranilate
HBI	Hydrogen Bond intensity
HBT	1-hydroxybenzotriazole
HMF	5-hydroxymethyl furfural
HMQC	Heteronuclear multiple quantum coherence
HPAEX	High-Performance anion exchange chromatography
HPLC	High-Performance liquid chromatography
HSQC	Heteronuclear single-quantum coherence
HWE	Hot water extraction

ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IR	Infrared
L/C	Lignin to cellulose ratio
LCB	Lignocellulosic biomass
LFAD	Lateral fibril aggregate dimension
LFD	Lateral fibril dimension
LHW	Liquid hot water
LiP	Lignin peroxidases
LOI	Lateral order index
MA1	Malt extract – agar medium
MM	Mineral media
MnP	Manganese peroxidases
NDF	Neutral detergent fibre
NMR	Nuclear magnetic resonance
NREL	National Renewable Energy Laboratory
NS	Nelson-Somogyi method
OPEFB	Oil palm empty fruit bunch
RS	Reducing sugars
S/G	Syringyl to Guaiacyl ratio
SEC	Size exclusion chromatography
SEM	Scanning electron microscope
SEW	Steam-exploded wood
SHF	Separate Hydrolysis and Fermentation
SL	Soda lignin
SmF	Submerged fermentation



SMS	Spent mushroom substrate
SMWS	Spent mushroom wheat straw
SSCF	Simultaneous saccharification and co-fermentation
SsF	Solid state fermentation
SSF	Simultaneous Saccharification and Fermentation
TAPPI	Technical Association of Pulp and Paper Institute
TC	Total Carbon
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TRS	Total reducing sugars
TS	Total solids
UV	Ultraviolet
VA	Veratryl alcohol
VFA	Volatile fatty acids
VP	Versatile peroxidases
VS	Volatile solids
WAXD	Wide-angle X-ray Diffraction
WRF	White-rot Fungi
WS	Wheat straw
XRD	X-ray diffraction

# **I General Introduction**

The global energy crisis in recent times has caused an unprecedented impact that will be felt for years to come. This has emphasized the need to accelerate the transition to a more sustainable and secure energy system. The push for energy resilience has a paradigm shift underway toward renewable energy (International Energy Agency, 2022). There has been a rapid increase in the number of governments making ambitious net-zero emissions by 2050 pledges. However, even though there is growth in low-carbon electricity, most of the world's shipping, aviation, and certain industrial sectors are not yet "electric-ready" and require other energy sources. This drives the greater demand for biogas and biomethane to gain a firmer foothold in global energy consumption. In 2018, global direct consumption of biogas was approximately 35 Mtoe (Million tons of oil equivalent) with Europe being the leading biogas-producing region. Currently, 70% of biomethane used in Europe is from energy crops. Therefore, policies that encourage the use of crop waste are gaining traction to discourage the food vs. fuel debate present while using energy crops. It is projected that the direct consumption of biogas will reach around 75 Mtoe in 2040. Most of it aims to be produced from centralized plants fed by agricultural and municipal solid wastes to meet the local power and heating demands. There is also a considerable motivation to develop biogas for clean cooking in Africa and India. Biogas production not only provides reliance for energy needs but also improves waste management practices (municipal solid waste, wastewater sludge, crop residues, animal manure) and also reduces air pollution (due to stubble burning and coal usage), especially in developing countries. Moreover, the upgradation of biogas into biomethane for use as a transport fuel is increasing in Europe and North America. The distinctive advantage of biomethane over bioethanol and biodiesel is based on blend share limitations since bioethanol and biodiesel are not identical to gasoline and diesel. However, biomethane can fully replace natural gas as a fuel source without any engine changes. Therefore, the transformation of organic waste such as agricultural residues into higher-value products such as biogas and biomethane fits well into the concept of circular economy (International Energy Agency, 2020).

Even though bioenergy is a promising renewable energy, there are some barriers to its deployment. Some of the factors that reduce the market demand are policy uncertainties and a lack of awareness of bioenergy products. The financial and economic barriers that come along with the complex institutional structures are the subsidies for fossil fuels that make bioenergy

not so competitive in the market. Therefore, carbon pricing policies and subsidies for bioenergy can make bioenergy more affordable. The major factors that deter the transition are weak supply chains and a low level of technology readiness. The unstable feedstock supply due to the lack of information on the availability and location of bioenergy resources causes weak supply chains. Poor regulations on quality control and standardization, lack of infrastructure, and very little research on large-scale bioenergy production are the causes of a low level of technology readiness. Hence, the right mix of policies, research, development, and demonstration can help to accelerate the commercialization of bioenergy (Feng, 2022).

Anaerobic digestion (AD) is a biological process that converts organic matter, such as energy crops, agricultural and forest residues, sewage sludge, and animal and food waste, into biogas (primarily methane and carbon dioxide). AD occurs in the absence of oxygen with the help of microorganisms and usually consists of several stages. The first stage is hydrolysis where complex organic molecules are broken into simpler sugars by enzymes secreted by microorganisms. In the second stage, acidogenesis, sugars are converted to organic acids such as acetic acid and propionic acid by acid-forming bacteria. This is followed by acetogenesis where the organic acids are converted into acetate, hydrogen, and carbon dioxide by acetogenic bacteria. Finally, as a fourth stage methanogenesis occurs where the acetate, hydrogen, and carbon dioxide are converted into methane and carbon dioxide by methanogens (Uçkun Kiran et al., 2016). Of the organic matter available, lignocellulosic biomass (LCB) is the most abundant and cheap renewable resource on Earth. Approximately,  $120 \times 10^9$  tons of LCB per annum are produced globally, which could produce  $2.2 \times 10^{21}$  Joule of energy which is 300 times more than the existing global energy requirement (Guo et al., 2015). Biomethane production from LCB is considered an efficient means of energy production because of a higher energy output-to-input ratio (Abraham et al., 2020).

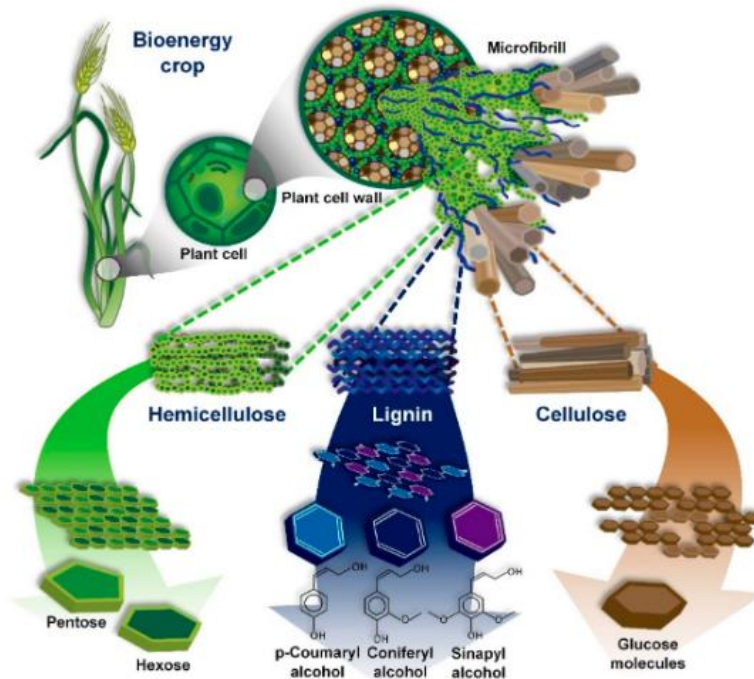


Figure I-1: Structure of lignocellulosic biomass and its biopolymers (Reuse (print) with permission of Hernández-Beltrán et al. (2019) (Creative Commons Attribution (CC BY) license)).

Lignocellulose is the major part of plant cell walls and is made up of three main macromolecules, namely, lignin, hemicellulose, and cellulose (as shown in Figure I-1). These three components form a complex and resistant three-dimensional structure via covalent and hydrogen bonding. The proportion of each component varies for each biomass (herbaceous plants, stems, and leaves of cereals, etc.) and also depends on the origin (source, species, climatic conditions, etc.) (Alfenore and Molina-Jouve, 2016; Anukam and Berghel, 2021). One of the major agricultural residues is straw since about 35 million metric tons of wheat are produced per year in France and about 0.85 kg of straw is generated per kg of wheat harvested (Zhang et al., 2022). The inherent characteristics of biomass such as high moisture content, low energy density, low bulk density, irregular shape, and size, make it difficult to use in its natural form for energy production. Moreover, the structural complexity and heterogeneity of biomass, the crystalline nature of its cellulose content, and the extent of lignification are responsible for the recalcitrant nature of biomass. For better exploitation of biomass, a pretreatment step is essential to overcome its recalcitrance, which is majorly caused by lignin. The pretreatment step helps to disintegrate lignin from cellulose and hemicellulose components (as shown in Figure I-2). This renders the cellulose accessible and increases the surface area for the microbial

and enzymatic attack which prepares the biomass for maximum product recovery. There are several methods of pretreatment, including physical, chemical, physicochemical, and biological methods. The choice of pretreatment depends on the type of LCB used and the downstream process (Alfenore and Molina-Jouve, 2016; Anukam and Berghel, 2021).

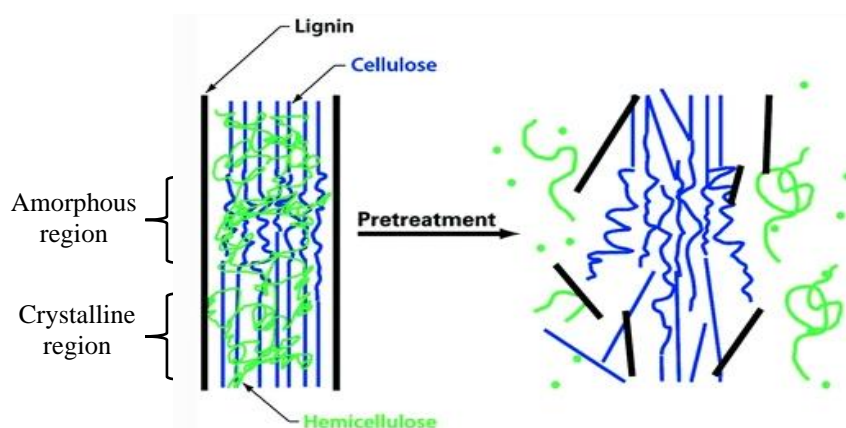


Figure I-2: Schematic representation of lignocellulosic biomass pretreatment (Adapted image for reuse (print) with permission of Ji et al. (2011) (Copyright Springer Nature)).

Physical pretreatment is inevitable prior to biochemical and/or thermochemical conversion of biomass in order to eliminate mass and heat transfer limitations. It improves the accessibility to polysaccharides of the biomass and increases the surface area for microbial attack. However, there is limited information on how physical pretreatment modifies the structure or the chemical composition of biomass. Some of the drawbacks of physical pretreatment are its lack of ability to get rid of lignin and its high energy consumption. Common physical methods include grinding and milling, while common chemical methods include acid, alkali, and oxidant treatments. Chemical pretreatment involves the use of organic or inorganic compounds to disrupt the biomass structure through interaction with intra- and inter-polymer bonds of primary organic compounds. Chemical pretreatment is a commonly used method because of its high efficiency. Nevertheless, it could significantly impact the downstream process owing to the high concentration of chemical reagents used. Moreover, additional processes are required to recycle the reagents used, else, the residue could cause environmental pollution. Physicochemical pretreatment is a combined approach, performed over a wide range of temperatures (from 50 to 250 °C) to break the hydrogen bonds between complex polymers using heat. The utilization of waste heat streams is important for efficient energy management. The pretreatment time in the physicochemical process needs to be optimized as prolonged heat

exposure can lead to unwanted reactions and formations of inhibitors to the AD process. On the other hand, biological pretreatment is a low-cost, environmentally friendly approach as it involves using enzymes or microorganisms to break down the biomass. It requires less energy and mild process conditions. It helps in delignification, decomposition of hemicellulose, and reduction of cellulose crystallinity. The disadvantage of biological pretreatment is the longer residence period and lower efficiency (Abraham et al., 2020; Anukam and Berghel, 2021; Mankar et al., 2021).

Despite lignin being a massive natural carbon and energy reservoir, only a small group of filamentous basidiomycete fungi called white-rot fungi (WRF) have evolved the ability to efficiently depolymerize and mineralize lignin to CO<sub>2</sub> and H<sub>2</sub>O. WRF uses extracellular ligninolytic oxidoreductase systems, such as laccases and peroxidases, along with other secreted metabolites for lignin depolymerization (del Cerro et al., 2021). However, the porosity of LCB is small in comparison to the size of ligninolytic enzymes, therefore, it is not able to penetrate the cell wall. Therefore, delignifying fungi use a non-enzymatic system called chelator-mediated Fenton reaction to carry out initial degradation as reactive oxygen species have a low molecular weight and can penetrate the cell wall (Hashemi et al., 2022). *Pleurotus ostreatus*, commonly known as oyster mushroom, is a WRF that is cultivated widely for its high nutritional and medicinal value. It can be grown on a wide range of substrates and the mycelium residues obtained after harvesting the edible mushroom are called spent mushroom substrate. However, fungal pretreatment has a long reaction time and low efficiency. Therefore, a combination with a chemical pretreatment that mimics the degradation of plant cell walls by fungal action, could improve the fungal pretreatment efficiency. Fenton pretreatment is an oxidation-reduction process of iron by H<sub>2</sub>O<sub>2</sub>. It is a cyclic reaction and the generated reactive hydroxyl radicals can contribute to the degradation of the plant cell wall. The fungal pretreatment requires very low energy while the chemical pretreatment used in combination would only need mild operating conditions, the combination improving the overall process productivity (Hashemi et al., 2022; Shirkavand et al., 2016). The aerobic fungi use up carbohydrates for their respiration and their pretreatment process is quite long. Contrarily, anaerobic fungi can prevent carbohydrate loss due to respiration (Vinzelj et al., 2020).

Anaerobic fungi produce cellulolytic enzymes that are able to cleave the ester bonds interlinking hemicellulose and lignin. They have synergistic and antagonistic relationships with other organisms such as methanogens which can consume the intermediate products.

Consequently, bioaugmentation of the AD system with anaerobic fungi can boost methane production (Vinzelj et al., 2020). Several genera such as *Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Cyllamyces*, *Piromyces*, *Anaeromyces*, and *Feramyces* were detected in agricultural biomass plants with a high share of cattle slurry. This shows that anaerobic fungi can be present and transcriptionally active in biogas plants but with substantial restrictions. Since their metabolic activity in biogas plants is scarce to low intensity, it is unclear which parameters impede its activity in AD (Flad, 2020). Some bioaugmentation studies have already been carried out using *Neocallimastix*, *Orpinomyces*, *Piromyces*, and *Anaeromyces* for biogas production. *Feramyces austinii* is a recently isolated strain from wild Barbary sheep and fallow deer. It was shown to have a fast and robust growth on plant biomass and the capability to metabolize a wide range of mono-, oligo-, and polysaccharides (Hanafy et al., 2018). Therefore, these relatively unexplored organisms are interesting for exploring their potential for biogas production. However, they provide a formidable challenge to scale the production process due to their still unknown complex growth requirements (Kazemi Shariat Panahi et al., 2022; Vinzelj et al., 2020).

It is also important to determine the quintessential physicochemical properties of biomass to better understand how suitable is the material for conversion and the effect of the pretreatment on biomass. By understanding the structural changes in the biomass, the mode of action of the pretreatment to boost the biogas yield can be ascertained. Some of the important factors of a successful pretreatment are lignin degradation, change in the cellulose degree of polymerization, and accessibility of the enzymes. Therefore, it is impossible to investigate the effect of pretreatment with just one factor. Nevertheless, heterogeneity is an inherent characteristic of biomass. Consequently, a wide variety of cutting-edge analytical techniques are needed to interpret biomass characteristics before and after pretreatment. Each analytical technique has its specific drawbacks which make it difficult to exactly predict the effectiveness of a pretreatment (Anukam and Berghel, 2021; Karimi and Taherzadeh, 2016). Consequently, it is important to carry out anaerobic digestion or enzymatic saccharification to understand the effect of pretreatment on the yield of desired biofuel.

## II Research Objectives and scientific approach

In this thesis, the effect of individual and combined pretreatment on straw was studied for its ability to enhance biogas production. Straw was used for this study because it is cheap and abundant biomass. A detailed bibliography of different fungal–chemical pretreatment was carried out based on their efficiency for fiber degradation, carbohydrate production, biogas, and bioethanol yields. This helped to choose the conditions and pretreatment strategies to be used. The scientific approach to achieve this aim was to first study the fungal and chemical pretreatment steps individually and choose the optimized conditions for the combinatorial pretreatment. For the biological pretreatment, *Pleurotus ostreatus* was chosen as 2500 tons of it is produced annually in France for consumption. Therefore, the spent mushroom substrates are a valuable resource for energy production. The mushroom production process occurs using solid-state cultivation. Even though solid-state cultivation provides an environment that filamentous fungi are naturally adapted to, submerged cultivation provides a more homogenous environment. One of the aims of this work was, therefore, to evaluate the biogas production after fungal pretreatment in solid-state and submerged cultivation. Different strains of *Pleurotus ostreatus* were used for this study, namely, hybrid strains from the mushroom industry and pure strains from the mycology bank. For chemical pretreatment, oxidant pretreatment using  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  was studied as it mimics the Fenton-like reaction naturally used by white-rot fungi. Generally, physical pretreatments like grinding and autoclaving are carried out before any pretreatment. Therefore, their effect on the straw structure and biogas production was also estimated. A wide range of analytical techniques was used to characterize the biomass before and after pretreatment to establish the correlation of biomass characteristics with its biomethane production. Preliminary research was also carried out on bioaugmentation with anaerobic fungi. The chosen strain *Feramyces austinii* was isolated and characterized only recently and therefore, this is one of the first studies of using this strain for AD enhancement. The overall scheme of the project is presented in Figure II-1.



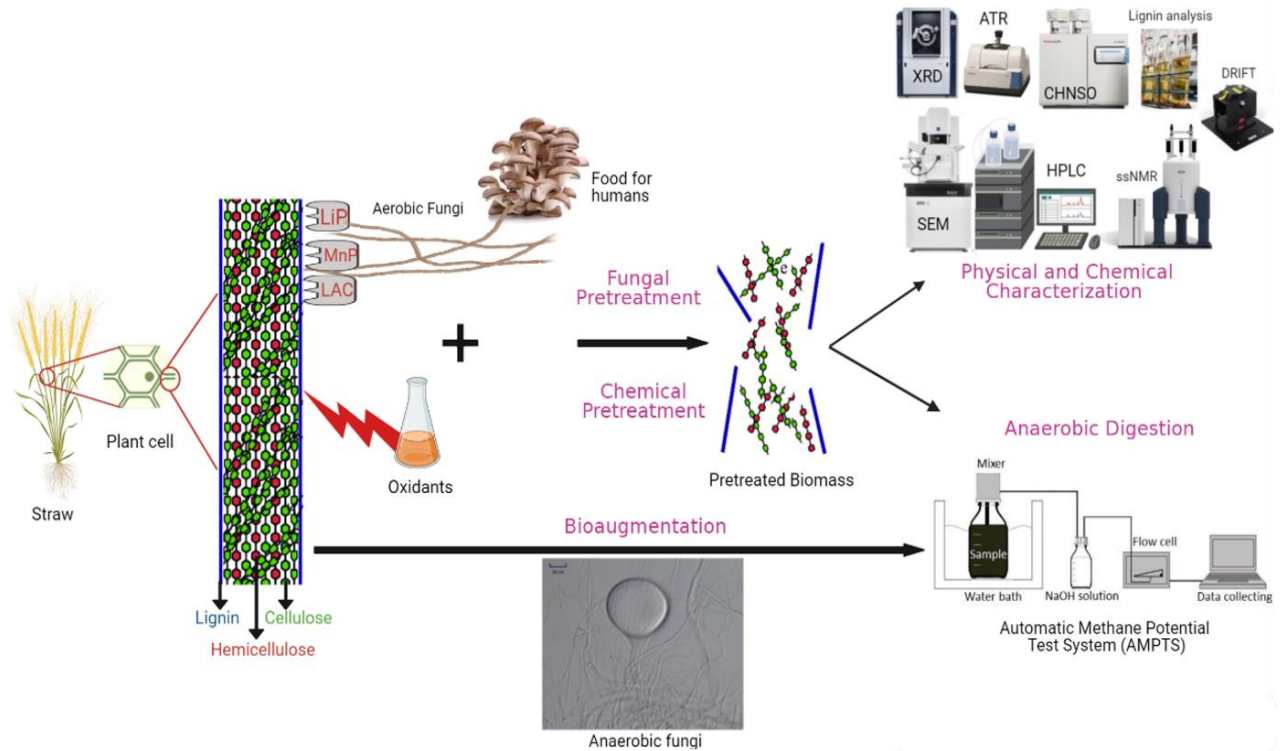


Figure II-1: Schematic representation of the overall project. Created with BioRender.com.

The scientific approach to this thesis consisted of various smaller goals as stated below:

1. An extensive review of combined biological-chemical pretreatment of LCB based on its fiber degrading ability, sugar production, biogas yield, and bioethanol yield. A review of various analytical techniques used for the characterization of biomass.
2. Optimization of oxidants concentration for pretreatment of straw based on literature.
3. The effect of particle size, autoclaving process, and Fenton reaction on biomethane production from straw.
4. Comparison of two spent mushroom substrates before and after different harvest stages for biogas production to enable a “waste-to-energy” approach for mushroom industries.
5. Comparison of different strains of *Pleurotus ostreatus* obtained from mycology bank based on its biomass degradability under submerged conditions.
6. Study of the influence of the order of combined fungal–oxidant pretreatment on biomass characteristics and for biogas production.
7. Preliminary study of bioaugmentation with a new strain of anaerobic fungi, *Feramyces austinii*, to study its effect on biogas production from straw.

These objectives stated above are presented in the manuscript in the following manner:

In Chapter III of this thesis, a review of the bibliography is presented as a collection of three published articles. The first article published in *Bioresource Technology* in 2021 discusses the efficiency of different combined biological and chemical/ physicochemical pretreatment on fiber degradation and sugar production. It was established that it is important to determine the order of pretreatment in combined strategies based on the mechanism of action of individual pretreatment methods. The second article published in *Applied Microbiology* in 2022 discusses the combined biological and chemical/ physicochemical pretreatment strategies studied so far for biogas and bioethanol production. Only the fungal/enzyme-alkaline pretreatment combination had been studied as part of biological–chemical sequential treatment of LCB for biogas production until the valorization of this article. The third part of this section is an extract from a book chapter that was published by Springer Nature in the book titled *Fungal Biopolymers and Biocomposites: Prospects and Avenues* in 2022. The enzymatic oxidative degradation of lignin by fungi is detailed here. The different analytical techniques used to characterize lignin are also discussed at length. This extensive review paved the way for choosing fungal – Fenton combined pretreatment of LCB to improve biogas production.

Chapter IV is devoted to the chemical pretreatment studies on straw. The first part of the chapter discusses the optimization of Fenton pretreatment based on simple characterization techniques. The second part is presented as a published article in *Energies* in 2023 which discusses at length the difficulties in biomass characterization. It presents a wide range of state-of-the-art analytical techniques used for biomass characterization to understand the action of physical and chemical pretreatment on biogas production.

Chapter V deals with the biological pretreatment of straw. In the first part of this chapter, the potential of spent mushroom substrate for biogas production is elucidated for developing an integrated food and energy biorefinery. The second part examines the variation in the efficiency of fiber degradation and carbohydrate production with different strains of *Pleurotus ostreatus* under submerged conditions.

In Chapter VI, the combined biological and chemical pretreatment with 2 strains of *Pleurotus ostreatus* (hybrid and pure) and Fenton reaction for biogas production is presented. The influence of the order of sequential pretreatment on biomass characteristics and biogas production is compared with sole fungal pretreatment.

Chapter VII presents a preliminary study of using anaerobic fungi to improve the biomethane yield. A new methodology of bioaugmentation with filamentous anaerobic fungi, *Feramyces austinii*, is experimented with and provides perspectives on the challenges faced in the scale-up of the production process of anaerobic fungi.

All the materials and methods related to each research objective are presented in the corresponding chapters. This thesis ends with a summary (Chapter VIII) of all the results obtained and states the limitations of the study and postulates the scope of future research.

## III Literature Review

### III.1 Fiber degradation and carbohydrate production by combined biological and chemical/ physicochemical pretreatment methods of lignocellulosic biomass – A review.

This literature review was published in Bioresource Technology in 2021 and is reprinted here with the editor's permission. An adapted version without the core of the text being modified is presented here and the references are provided in Chapter X.

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### Fiber degradation and carbohydrate production by combined biological and chemical/physicochemical pretreatment methods of lignocellulosic biomass – A review

S. Meenakshisundaram<sup>a</sup>, A. Fayetulle<sup>a</sup>, E. Leonard<sup>a</sup>, C. Ceballos<sup>a</sup>, A. Pauss<sup>a,\*</sup>

<sup>a</sup> Université de technologie de Compiègne, ESCOM, TIMR (Integrated Transformations of Renewable Matter), Centre de recherche Royallieu - CS 60 319, F-60 203 Compiègne Cedex, France

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#### HIGHLIGHTS

- Pretreatment is an essential step for the breakdown of lignocellulosic biomass.
- Combining pretreatment helps to lower the severity of the drawbacks of a single step.
- Biological-chemical/physicochemical combined pretreatment strategies were reviewed.
- The order of pretreatment in the combined studies affects the overall efficiency.

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#### ARTICLE INFO

**Keywords:**  
Lignocellulosic biomass  
Combined pretreatment  
Fiber degradation  
Sugar yield

#### ABSTRACT

Sustainable biorefinery concepts based on lignocellulosic biomass are gaining worldwide research interest because of their inexpensiveness and abundance. The recalcitrance of lignocellulosic biomass poses a major hindrance to enhance biofuel production. Therefore, a pretreatment step is critical to prepare the substrates for the downstream process. Combining pretreatment steps help to lower the severity of the drawbacks of a single pretreatment step. This paper systematically reviews the combined biological and chemical/physicochemical pretreatment based on fiber degradation and sugar yield. An energy-efficient biological pretreatment method combined with a chemical pretreatment that accelerates the pretreatment times has been seen to be efficient for fiber degradation and sugar yields. However, fungal species, culture conditions, biomass type, the severity of chemical pretreatment and the order of sequential pretreatment influences the relative component contents and sugar yield. Even the same biomass from different sources undergoing similar pretreatment conditions could result in a varying amount of digestibility.

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#### III.1.1 Introduction

The energy crisis is receiving worldwide attention and the current need is to fulfill the ever-growing energy demand sustainably. It is not only enough to produce energy, heat, and

transport fuel but also to increase the security of the energy supply. Improving energy security is essential to transition out of conventional energy (Ošljaj and Muršec, 2010). The move towards renewable energy has to be done without diverting land use or food crops for the production of energy (Tomei and Helliwell, 2016). Lignocellulosic biomass (LCB) resources like energy crops, agriculture, and forest residues are abundant and are good renewable feedstocks for bioenergy production. It is assessed that the annually produced terrestrial biomass stores 3-4 times greater energy than the existing global energy demand (Guo et al., 2015). In the last decade, the debate of food and land use versus fuel has been raised with the assumption that a crop has a single utility. Many feedstocks have multiple uses including human food consumption, animal feed, industrial applications, and the generation of energy (Tomei and Helliwell, 2016). Generally, crop residues from cereals such as rice and wheat are mainly used as fodder and for manure applications. The surplus unutilized crop residues are openly burned in most of the developing countries which is a major cause of air pollution (Sukumaran et al., 2010). Therefore, the safe disposal of waste is another biggest challenge to humankind. Combining these challenges, it is possible to utilize the full potential of organic waste to produce energy and reduce the dependence on fossil energy resources (Ošljaj and Muršec, 2010).

Biomass is a theoretically viable and economical source of renewable energy carrier for the production of bio-oil, biogas, biodiesel, and bioethanol using a wide range of technologies. Over the last decade, there have been numerous researches to produce biofuels from lignocellulosic feedstock (Valdivia et al., 2016). However, the physical and chemical structural rigidity and recalcitrance nature of lignocellulose has made it difficult and highly expensive to produce sugars from carbohydrates in lignocellulose (Mosier, 2005). The major components of lignocellulosic biomass are lignin, hemicelluloses, and cellulose. The substrates enabling biofuel production are sugars contained in cellulose and hemicelluloses but are protected by the resistant structure of lignin. Therefore, a pretreatment step is required before the downstream process to break the lignin seal and reduce the overall crystallinity of the biomass structure so that the surface area for enzyme accessibility and microbial attack can be increased. The microbial breakdown of polymer chains of cellulose and hemicellulose will help to increase the rate of biomass degradation and help to convert the fermentable sugars into biofuel (Anwar et al., 2014).

The pretreatment process has to be chosen based on the techno-economic feasibility of integrating into the downstream process with considerations of configurations and efficiency of downstream operations (Zheng et al., 2014). A mechanical pretreatment step such as a hammer mill will help to break the tubular structure and reduce the size, which will prevent floatation. It has been observed that mechanical pretreatment provides a minimal improvement in biogas production but the high energy demand of the process makes it an expensive addition to the AD process (Kratky and Jirout, 2011). Chemical pretreatment uses either acid, alkali, oxidants, or organo-solvents and each chemical uses a different mode of action to efficiently remove the lignin or hemicelluloses present in the biomass (Abraham et al., 2020). In comparison to physical and biological pretreatment methods, chemical methods have a better degradation effect and faster rate of degradation of the complex lignocellulosic structure. Although chemical pretreatment is the majorly investigated strategy, the disadvantages of this method are also significant. For example, acid pretreatment using sulfuric acid ( $\text{H}_2\text{SO}_4$ ) and nitric acid ( $\text{HNO}_3$ ) can increase the content of  $\text{H}_2\text{S}$  and  $\text{N}_2$  in biogas, which results in the need for additional gas cleaning and thereby increasing the investment costs. In a study where several chemical and physicochemical pretreatment methods for biogas production from wheat straw were compared, the authors found none of them to be cost-effective as additional chemicals or high energy were required. Albeit NaOH pretreatment being proven as an efficient and significantly cheaper pretreatment method for biogas yield in AD, the probability of  $\text{Na}^+$  ion inhibition for methanogenesis is higher. Besides, disposal of digestate containing  $\text{Na}^+$  is difficult as it causes soil salinization (Zheng et al., 2014). A method to solubilize lignocellulosic components efficiently without the formation of inhibitors is the physicochemical method. This includes steam explosion, hydrothermal, and Ammonia Fiber Explosion (AFEX). However, this pretreatment step which exposes the lignocellulosic structure for hydrolysis based on the temperature and moisture content and which produces higher yield in the subsequent bioprocesses is an expensive method because of the high energy needs (Hernández-Beltrán et al., 2019). Biological pretreatment using fungal, microbial consortium and enzymes is an inexpensive and more sustainable strategy. While the advantages of biological pretreatment include substrate and reaction specificity, low energy requirements, and no generation of toxic compounds, the disadvantages are relatively low efficiency, a considerable loss of carbohydrates, and long residence periods (Zheng et al., 2014).

From the highlights and challenges of pretreatment methods discussed, it can be observed that although single pretreatment makes a significant contribution, no single method provides efficient results with its intrinsic limitations. Therefore, combined pretreatment strategies could lower the severity of the disadvantages and provide the desired result (Zheng et al., 2014). For example, the combination of microbial and chemical pretreatments is perceived to shorten the pretreatment times, reduce the strength of chemicals used and thereby the secondary pollution associated with it, and as a cost-effective strategy. While pretreatment by physical, chemical, and biological methods has been studied extensively, the combined pretreatment strategies are gradually being developed in recent years for their synergistic effect. Physical and chemical combined pretreatment is a more commonly used combined pretreatment method but the combination of biological and chemical pretreatment is yet to be well studied (Shirkavand et al., 2016).

Therefore, the aim of this work is to present an updated review of combined microbial-chemical/physicochemical pretreatment strategies used for different LCBs based on fiber content degradation (Subchapter III.1.2) and sugar yield (Subchapter III.1.3).

### **III.1.2 Use of fiber content degradation analysis to evaluate the combined pretreatment effect**

The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. These form the highly ordered crystal structure of the plant cell wall, which causes heterogeneity, and the recalcitrant nature of the lignocellulosic biomass. The relative quantity of the three major components varies widely among the various biomasses (40–50% cellulose, 25–30% hemicellulose, 15–20% lignin) and amongst the same biomass depending on its cultivation and harvesting conditions. The minor compounds are usually proteins, starches, pectins, tannins, etc., and are called extractives (Adjalle et al., 2017; Chen et al., 2017). The structure of lignocellulose is such that cellulose microfibrils are integrated into the hemicelluloses matrix and covalently cross-linked with the heterogeneous lignin. The main fraction that causes recalcitrance of the lignocellulosic biomass is lignin, which is made up of non-linear phenolic polymer built with chemically diverse and poorly reactive linkages. Lignin is relatively hydrophobic and aromatic (Cesarino et al., 2012; Paudel et al., 2017). Klason lignin, also known as acid-insoluble lignin (AIL) is the most abundant lignin content in most lignocellulosic biomass and is the insoluble residue portion after removing the ash by

concentrated acid hydrolysis of the biomass. On the other hand, the acid-soluble lignin (ASL) fraction that is soluble in 72% sulfuric acid is the reminder fraction. The sum of ASL and AIL is used to determine the total lignin content (Technical Committee ISO/TC, 2020). Hemicellulose is a heterogeneous polysaccharide and is non-covalently bonded (weakest bonded) to the surface of the cellulose fibrils and forms an amorphous matrix. It is thermo-chemically sensitive and includes arabinoxylan, glucomannan, glucuronoxylan, xylan, and xyloglucan. Xylan is the main component of hemicelluloses, which contains C5 sugars, C6 sugars, and sugar acids. Cellulose is the major fraction of lignocellulose and is made up of linear (1-4)  $\beta$ -D-glucan, which is a glucose polysaccharide. Albeit its large size, crystalline cellulose is hydrophilic. The intermolecular and intramolecular hydrogen bonds provide the strength to cellulose by forming a crystalline and amorphous structure (Paudel et al., 2017). In some papers reviewed in this chapter, xylan content is taken as a measurement of hemicellulose fraction while glucan content is considered as a measurement of cellulose fraction of biomass.

During a pretreatment process, changes occur in the microstructure, macrostructure, and chemical composition of lignocellulose. Lignin is broken down and removed, hemicelluloses are degraded and the crystalline structure of cellulose is changed (Paudel et al., 2017). A detailed characterization of the fiber content will help to determine the nature of lignocellulosic biomass, characterize the biodegradability properties of a pretreatment method, and to estimate the biofuel yield. Many such characterization methods for component analysis have been developed with specific applications and industries in mind. The most widely cited method for application in second-generation biofuels and chemicals is “Determination of structural polysaccharides and lignin in biomass” by Browning (1967) provided by National Renewable Energy Laboratory (NREL) (Karimi and Taherzadeh, 2016). NREL procedure is a two-stage method, where the first stage is a time-consuming process of removal of non-structural components using both water-soluble and ethanol-soluble extraction materials. The second stage involves using strong sulfuric acid to hydrolyze the polymeric carbohydrates to monomers and determination of the monomers using High-Pressure Liquid Chromatography (HPLC) (Mourtzinis et al., 2014). For forage and feed analysis in which digestible fiber is the most desired fraction, the Van Soest method (Soest and Wine, 1967) and the Association of Official and Analytical Chemists International (AOAC) standards are used. The AOAC standards cannot measure all non-digestible carbohydrates and therefore, the Van Soest method is preferred (Agblevor and Pereira, 2013; Theander et al., 1995). The Van Soest method is also



known as the Neutral Detergent Fiber (NDF) method as it is based on extracting the soluble fraction quickly using a neutral detergent, followed by the extraction of the insoluble part, i.e., lignin using an acid detergent. The lignin is determined using the Klason method (Mourtzinis et al., 2014). According to Mourtzinis et al. (2014), the Van Soest method is less time-consuming and more cost-effective than the NREL method, whereas the reliability of the NREL procedure was better. This is because the Van Soest method underestimates the lignin content and overestimates cellulose due to the long hydrolysis during the acid detergent lignin (ADL) determination step. Van Soest method also overestimates the hemicelluloses content as compared to the NREL method because extractives not solubilized during the NDF step will be solubilized during the acid detergent fiber (ADF) step (Adjalle et al., 2017). For analysis of woody biomass in which cellulose is the most desired fraction, the Technical Association of Pulp and Paper Institute's (TAPPI) procedure is used (Agblevor and Pereira, 2013). Browning (1967) estimated lignin using nitro-benzene oxidation method and quantifying the yield of vanillin or vanillin plus syringaldehyde (Kirk and Obst, 1988). The other methods used for fiber determination by the articles discussed in this chapter are the Iiyama and Wallis (1988) (perchloric acid method), Kaar and Brink (1991), Updegraff (1969), and Wise et al. (1946). Whichever method is used, care should be taken for sampling, particle size, moisture content, and presence of debris as it can affect the results. Though these different methods of analysis lead to wide compositional variation reported in the literature for the same biomass (Karimi and Taherzadeh, 2016), it is effective to study the fiber degradation of the combined pretreated lignocellulose to that of untreated biomass for the 23 articles discussed in Table III-1.

**Table III-1:** Fiber content degradation in different biological-chemical/physicochemical pretreatment strategies

Substrate	1 <sup>st</sup> step	2 <sup>nd</sup> step	Analytical technique	Lignin degradation (%)	Hemicellulose degradation (%)	Cellulose degradation (%)	Reference
<b>Biological – Alkaline Pretreatment</b>							
Corn stalks	<i>Irpe</i> x lacteus (28°C, 15 d)	0.25 M NaOH solution (75°C, 2 h)	NREL (Sluiter et al., 2006)	80	51.37	6.62*	Yu et al. (2010a)
<i>Pinus Radiata</i>	<i>Gloeophyllum trabeum</i> (27°C, 28 d)	25% w/ w NaOH (180°C, 5 h)	TAPPI 204 cm-97	37.5*	75.41*	N/A	Fissore et al. (2010)
<i>Populus tomentosa</i>	<i>Trametes velutina</i> D10149 (28°C, 56 d)	2.5 g NaClO <sub>3</sub> and 2 mL acetic acid (80°C, 1 h)	NREL (Sluiter et al., 2008)	72.75*	7.1*	(-) 11.42*	Wang et al. (2013a)
<i>Populus tomentosa</i>	<i>Trametes velutina</i> D10149 (28°C, 28 d)	70% (v/v) ethanol aqueous solution containing 1% (w/v) NaOH (75°C, 3 h)	Klason method (Dence, 1992; KCL, 1982)	23.08*	22.22*	18.91*	Yang et al. (2013)

Rice Straw	<i>Sphingobacterium</i> sp. LD-1 (30°C, 4 d)	4% NaOH + 6% Urea (-10°C, 4 h)	NREL (Sluiter et al., 2008)	34.38*	28.20*	(-) 34.81*	Dai et al. (2015)	
Willow sawdust	<i>Leiotrametes menziesii</i> (27°C, 30 d)	1% (w/v) NaOH (80°C, 24 h)	NREL (Sluiter et al., 2008)	59.8	68.1	51.2	Alexandropoulou et al. (2017)	
	<i>Abortiporus biennis</i> (27°C, 30 d)			54.2	51.8	29.1		
Rice Straw	70 mL of ethanol–water solution (65:35, v/v) containing 0.5 wt % NaOH, 400 W of microwave irradiation for 10 min	<i>Acinetobacter</i> sp. B-2 (30°C, 2 d)	(Teramoto et al., 2008)	51.76*	33.44*	(-) 32.80*	Si et al. (2019)	
				<i>Bacillus</i> sp. B-3 (30°C, 2 d)	53.58*	31.53*		(-) 28.39*
				<i>Pandoraea</i> sp. B-6 (30°C, 2 d)	59.95*	19.65*		(-) 21.06*
				<i>Comamonas</i> sp. B-9 (30°C, 2 d)	59.04*	22.98*		(-) 25.09*
<b>Biological – Acid Pretreatment</b>								

<i>Populus tomentosa</i>	<i>Trametes velutina</i> D1014 (28°C, 56 d)	1% sulphuric acid (140°C, 1 h)	NREL (Sluiter et al., 2008)	23.82*	75.96*	(-) 18.74*	Wang et al. (2013a)
Oil palm empty fruit bunches	<i>Pleurotus floridanus</i> LIPIMC996 (31°C, 28 d)	Ball milled at 29.6/s for 4 mins. Phosphoric acid treatment (50°C, 5 h)	NREL (Sluiter et al., 2011).	(-) 8.29	60.63	(-) 37.52	Ishola et al. (2014)
Olive tree pruning	<i>Ulocladium</i> sp. (23°C, 28 d)	0.1% sodium hydroxide (5% w/w) at (50°C, 1 h, 165 rpm); 0.5% (w/w) H <sub>2</sub> SO <sub>4</sub> (130°C, 1 h)	NREL (Sluiter et al., 2008)	(-) 61.70*	(-) 28.87*	(-) 36.00*	Martín-Sampedro et al. (2017)
	<i>Hormonema</i> sp. (23°C, 28 d)			(-) 62.23*	(-) 24.65*	(-) 37.78*	
	<i>Trametes</i> sp. (23°C, 28 d)			(-) 57.98*	(-) 33.10*	(-) 50.22*	
Rice straw	0.5% H <sub>2</sub> SO <sub>4</sub> (121°C, 40 mins)	<i>Cupriavidus basilensis</i> B-8 (30°C, 3 d)	(Teramoto et al., 2008)	(-) 91.72*	67.03*	(-) 47.13*	Yan et al. (2017)
Olive tree biomass	2% w/v H <sub>2</sub> SO <sub>4</sub> (130°C, 1.5 h)	<i>Irpex lacteus</i> (Fr.238 617/93) (30°C, 28 d)	NREL (Sluiter et al., 2010)	(-) 116.02*	73*	(-) 90.48*	Martínez-Patiño et al. (2018)

Olive tree biomass	<i>Irpex lacteus</i> (Fr.238 617/93) (30°C, 28 d)	2% w/v H <sub>2</sub> SO <sub>4</sub> (130°C, 1.5 h)	NREL (Sluiter et al., 2010)	(-) 105.82*	75.29*	(-) 69.52*	Martínez-Patiño et al. (2018)
Rice Straw	70 mL of ethanol–water solution (65:35, v/v) containing 0.5 wt % HCl, 400 W of microwave irradiation for 10 min	<i>Acinetobacter</i> sp. B-2 (30°C, 2 d)	(Teramoto et al., 2008)	31.59*	46.64*	(-) 37.49*	Si et al. (2019)
		<i>Bacillus</i> sp. B-3 (30°C, 2 d)		34.32*	38.06*	(-) 30.87*	
		<i>Pandoraea</i> sp. B-6 (30°C, 2 d)		49.83*	28.05*	(-) 29.40*	
		<i>Comamonas</i> sp. B-9 (30°C, 2 d)		34.32*	34.25*	(-) 26.83*	
<b>Biological - Oxidative Pretreatment</b>							
Rice Hull	H <sub>2</sub> O <sub>2</sub> (2%, 48 h)	<i>Pleurotus ostreatus</i> (28°C, 18 d)	(Goering and Van Soest, 1970)	37.54*	54.42*	11.92*	Yu et al. (2009)

Corn Straw	<i>Echinodontium taxodii</i> (25°C, 15 d)	0.0016% NaOH and 3% H <sub>2</sub> O <sub>2</sub> (25°C, 16 h)	Procedures of AOAC (Theander et al., 1995)	52.00	23.64*	(-) 45.45*	Yu et al. (2010b)
<i>Populus tomentosa</i>	<i>Fomitopsis palustris</i> (28°C, 28 d)	FeCl <sub>3</sub> (180°C, 30 mins)	NREL (Sluiter et al., 2008)	(-) 106.07*	99.7	27.81*	Wang et al. (2013b)
	<i>Trametes orientalis</i> (28°C, 28 d)			(-) 63.60*	98.8	2.88*	
Hemp chips	<i>Pleurotus eryngii</i> (28°C, 21 d)	3% NaOH and 3% (v/v) H <sub>2</sub> O <sub>2</sub> (40°C, 24 h)	TAPPI (1975)	55.7	23.2	25.1	Xie et al. (2017)
Rice Straw	0.02 M FeCl <sub>3</sub> , 1.5 M H <sub>2</sub> O <sub>2</sub> (25°C, 2 h)	<i>Cupriavidus basilensis</i> B-8 (30°C, 2 d)	Holocellulose (Wise et al., 1946); Cellulose (TAPPI 203); Klason lignin (Browning, 1967)	67.05*	21.34*	30.10*	Zhang et al. (2018)
Rice Straw	70 mL of ethanol–water solution	<i>Acinetobacter</i> sp. B-2 (30°C, 2 d)	(Teramoto et al., 2008)	36.79*	21.52*	(-) 11.50*	Si et al. (2019)

	(65:35, v/v) containing 0.5 wt % FeCl <sub>3</sub> . 400 W of microwave irradiation for 10 min	Bacillus sp. B-3 (30°C, 2 d)		34.04*	11.95*	(-) 5.96*	
		<i>Pandoraea</i> sp. B-6 (30°C, 2 d)		45.04*	(-) 5.27*	0.69*	
		<i>Comamonas</i> sp. B-9 (30°C, 2 d)		30.38*	1.91*	(-) 4.48*	
<b>Biological – Organosolv Pretreatment</b>							
<i>Pinus radiata</i> woods chips	<i>Ceriporiopsis subvermispora</i> (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 11,360); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)	TAPPI 222 om-88	76.17*	57.89* (Xylan)	(-) 86.06* (Glucan)	Muñoz et al. (2007)
<i>Acacia dealbata</i>	<i>Ganoderma australe</i> (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 10,920); cold alkaline wash: 1% NaOH	TAPPI 222 om-88	90.55*	78.76* (Xylan)	(-) 85.74* (Glucan)	Muñoz et al. (2007)

woods chips		for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)					
Sugarcane straw	<i>Ceriporiopsis subvermispora</i> (27°C, 15 d)	Acetosolv pulping (Acetic acid with 0.3% w/w HCl) (120°C, 5 h)	Lignin (Rocha et al., 1993); Hemicellulose and cellulose (Rocha et al., 1997)	86.8	93.8	32.1	Saad et al. (2008)
<i>Pinus radiata</i>	<i>Gloeophyllum trabeum</i> (27°C, 28 d)	60% ethanol in water solvent (200°C, 1 h)	TAPPI 204 cm-97	74.26*	80.74*	N/A	Fissore et al. (2010)
Pinus radiata wood chips	<i>Gloeophyllum trabeum</i> (ATCC 11539) (25°C, 21 d)	95% ethanol in water solvent (60:40 v/v ratio) with 0.13% H <sub>2</sub> SO <sub>4</sub> (w/v) (185°C, 18 min); 1092 H factor	TAPPI T280 pm 99	40.73*	91.58*	(-) 76.18* (Glucan)	Monrroy et al. (2010)
Pinewood chips	<i>T. versicolor</i> (28 °C, 15 d)	65% ethanol in water solvent with 1% H <sub>2</sub> SO <sub>4</sub> (v/v) (170°C, 1 h)	NREL (Sluiter et al., 2008)	N/A	N/A	17.1* (Glucan)	Kandhola et al. (2017)



Biological – LHW/ HWE/ Autohydrolysis Pretreatment							
Soybean	Liquid Hot water (170°C, 3 mins, 400 rpm, 110 psi, solid to liquid ratio of 1:10)	<i>Ceriporiopsis subvermispota</i> (28°C, 18 d)	NREL (Sluiter et al., 2008)	36.69	41.34	0.84	Wan and Li (2011)
Corn stover				41.99	42.91	7.09	
Wheat straw	Hot water extraction (HWE) (85°C, 10 mins, solid to liquid ratio of 1:20).	<i>Ceriporiopsis subvermispota</i> (28°C, 18 d)	NREL (Sluiter et al., 2008)	24.87	13.19	1.86	Wan and Li (2011)

\*as calculated by authors using the data given in the research article

(-): represents the increase in the content of the fiber.

### III.1.2.1 Biological – Alkaline Pretreatment

Biological-alkaline combination has been the most commonly studied combined pretreatment for lignocellulosic biomass so far. Of the seven research articles discussed in Table III-1, six of them have studied biological pretreatment followed by alkaline pretreatment while only Si et al. (2019) have studied the opposite sequential treatment. Biological pretreatment is preferred as the first step as this helps in the delignification of lignocellulosic fibers, which leads to a reduced concentration of alkali needed for the pretreatment of the substrate. An advantage of alkali pretreatment is that many of the caustic salts used can be regenerated (Zhong et al., 2011). The most common alkali pretreatment process is using NaOH as it causes a delignification reaction and decreases cellulose crystallinity. This results in an increase in surface area and enhanced enzymatic hydrolysis (Zhao et al., 2008). Zhong et al. (2011) state that NaOH pretreatment needs to be carried out at elevated temperatures (around 100°C) to have a satisfactory lignin degradation rate and sugar yield. The combination with biological treatment can enhance delignification and help lower the temperature of alkaline treatment, thereby reducing heating costs. Yu et al. (2010a), Yang et al. (2013), and Alexandropoulou et al. (2017) studied white-rot fungal treatment combined with NaOH treatment at around 75-80°C. Although the fungal pretreatment helped to either lower the temperature or shorten the duration of alkaline pretreatment (Yu et al., 2010a), this combination also resulted in a higher loss of carbohydrates (Alexandropoulou et al., 2017; Yang et al., 2013; Yu et al., 2010a). Wang et al. (2013a) studied white-rot fungus, *Trametes velutina* in combination with chlorite pretreatment at 80°C. Fungal pretreatment degraded lignin and hemicellulose partially while subsequent chlorite pretreatment greatly enhanced delignification compared to hemicellulose degradation. Beyond a threshold level of lignin degradation, Wang et al. (2013a) found out that lignin did not have an effect on cellulose conversion as either lignin was no longer a hindrance to enzyme attack or extensive delignification caused the lignocellulosic pores to collapse and thereby reducing the available surface area for enzyme adsorption (Zhu et al., 2008). On the contrary, Fissore et al. (2010) used a brown rot fungus, *Gloeophyllum trabeum*, in combination with NaOH treatment at high temperatures of 180°C. The brown rot fungus, as is well-known, decayed carbohydrates extensively compared to lignin. The alkaline medium at high temperatures also favored peeling and hydrolysis reaction of the carbohydrates and thereby lower lignin degradation (Fissore et al., 2010). While complete delignification is not necessary, a higher amount of delignification is required for carbohydrates conversion than hemicellulose

removal (Wang et al., 2013a). Use of bacteria in LCB pretreatment is gaining interest in the recent years as it can be rapidly grown and easier genetic manipulation is feasible as opposed to fungi. Dai et al. (2015) and Si et al. (2019) used bacteria in sequential pretreatment with milder alkaline pretreatment, which helped to shorten the overall pretreatment time of rice straw as compared to fungi. Dai et al. (2015) used a combination of NaOH and urea for chemical pretreatment as NaOH helps to break the inter- and intra- hydrogen bonds between cellulose molecules while urea acts as a hydrogen bond donor and receptor between solvent molecules, thereby preventing the reassociation of cellulose molecules and causing cellulose depolymerization. Though the combined bacterial-alkaline pretreatment did not enhance delignification greatly as compared to sole pretreatment, it significantly enhanced the saccharification of rice straw due to the increased content of cellulose and decreased content of hemicellulose in both Dai et al. (2015) and Si et al. (2019).

### **III.1.2.2 Biological – Acid Pretreatment**

Dilute acid pretreatment is known best for solubilization of hemicellulose fraction (Dai et al., 2015; Si et al., 2019) and this can be observed in all the six articles discussed in Table III-1 on biological-acid pretreatment. To reduce the severity of the acid pretreatment and the consequential inhibitory compounds formation, a combination with biological pretreatment is preferred. Martinez-Patiño et al. (2018) studied fungal pretreatment followed by dilute acid pretreatment and the converse sequential pretreatment for olive tree biomass. Though both the sequential pretreatment resulted in similar fiber degradation, the glucose and ethanol yield from fungal pretreatment followed by dilute acid pretreatment were much more remarkable. Wang et al. (2013a), Ishola et al. (2014), and Martin-Sampedro et al. (2017) also studied fungal pretreatment followed by dilute acid pretreatment. It can be observed in all three cases that while there was almost complete solubilization of hemicellulose, there was not substantial lignin removal in the combined pretreatment, resulting in cellulose being preserved. Similar results were obtained when Si et al. (2019) studied acid pretreatment in combination with bacterial pretreatment. In the study by Ishola et al. (2014) and Martinez-Patiño et al. (2018), it can be seen that percentages of cellulose and lignin are lower in the raw material (irrespective of the order of pretreatment steps) because the pretreatment methods removed at least most of the extractive fraction and thereby increasing the proportion of the rest of the components. The increase in lignin content in the study by Yan et al. (2017) was ascribed to the high temperatures at which the chemical pretreatment was conducted. SEM study showed that high temperatures

caused lignin to expand and become mobile while the aqueous environment triggered the lignin molecules to coalesce and form droplets on the surface of rice straw. The bacteria *Cupriavidus basilensis* B-8 remarkably acted as a scavenger, was bound to active sites on lignin droplets and used laccase to modify lignin morphology from droplets to creaks with flexible edges. The laccase uses H atom extraction mechanism to form a reactive phenoxy radical which helps to undergo further enzymatic or non-enzymatic reactions. The authors believe that this modification of lignin and laccase mechanism changes the hydrophobicity and subsequently the polarity on the surface, leading to accessibility to cellulose and reduced nonspecific binding of the cellulase enzyme (Palonen and Viikari, 2004; Yan et al., 2017). By and large, the combination of biological-dilute acid pretreatment did not yield higher saccharification as compared to sole pretreatment.

### **III.1.2.3 Biological - Oxidative Pretreatment**

With the understanding of the action of white-rot fungi in the biodegradation process, it helps to overcome the limitations in traditional bio-treatment like long residence time and ineffective delignification. The lignocellulose biodegradation mechanism by white-rot fungi involves a Fenton-based oxidation reaction. Therefore, mimicking the Fenton reaction-induced decay using oxidizing reagent like Hydrogen peroxide ( $H_2O_2$ ), will help enhance delignification without generating inhibitory by-products and unreacted chemical residues (Eastwood et al., 2011; Paudel et al., 2017). Six research articles have been compared for fiber degradation by biological-oxidative pretreatment of which four studies used fungi and two studies used bacteria for biological pretreatment. In the study by Yu et al. (2009), the oxidative treatment ( $H_2O_2$ ) followed by fungal pretreatment (*Pleurotus ostreatus*) was very effective as the delignification rate doubled and the carbohydrates losses reduced compared to that of sole biological treatment. The fungal pretreatment time reduced from 60 days for sole pretreatment to 18 days for combined pretreatment as structural changes during the oxidative pretreatment allowed for rapid penetration of fungal hyphae into the feedstock. *T.versicolor* showed the highest lignin-degrading ability amongst *Ganoderma lucidum* and *Echinodontium taxodii* in the sole fungal pretreatment studied by Yu et al. (2010b) but it also resulted in high cellulose loss, which subsequently resulted in low sugar yield. Therefore, the authors preferred *E. taxodii* to be used for combined pretreatment as cellulose loss was significantly lower. Yu et al. (2010b) and Xie et al. (2017) carried out oxidative pretreatment in alkaline conditions because  $H_2O_2$  produces hydroxyl and superoxide radicals which are very reactive at high pH (Paudel et al., 2017). The

combined white-rot fungi-alkaline/oxidative pretreatment resulted in significant enhancement of delignification compared to individual pretreatment, which helped to decrease the unproductive adsorption of cellulase in the subsequent enzymatic hydrolysis step. Wang et al. (2013b) studied both a brown rot fungus (*Fomitopsis palustris*) and a white-rot fungus (*Trametes orientalis*) in combination with FeCl<sub>3</sub>. It is very evident from the results that compared to the white-rot fungus, brown-rot fungus selectively degrades carbohydrates in lignocellulosic materials without removing the surrounding lignin. This is possible because the lignin is solubilized at temperatures (operating temperature of 180°C) higher than the temperature range of lignin phase transition. This causes the lignin molecules to coalesce into large bodies and migrate out, which then get redeposited on the cell walls causing an increase in the amount of lignin (Donohoe et al., 2008; Liu et al., 2009). The tremendous hemicelluloses degradation was attributed to the ability of Fe<sup>3+</sup> ion in FeCl<sub>3</sub> to act as good electron acceptor capability and synchronize with the oxygen donor atoms of carbohydrates to hydrolyze hemicelluloses (Yu et al., 2011). This masked the synergy of the combined biological-oxidative pretreatment in hemicelluloses degradation (Wang et al., 2013b). Zhang et al. (2018) and Si et al. (2019) conducted oxidative pretreatment followed by bacterial pretreatment on rice straw. Ligninolytic bacteria such as *Cupriavidus basilensis* and *Pandoraea sp.* showed the outstanding potential to selectively remove lignin while the saccharolytic bacteria *Acinetobacter sp.* easily utilized amorphous hemicelluloses (Si et al., 2019; K. Zhang et al., 2018). In the study by Zhang et al. (2018), the free radicals produced by the Fenton reaction resulted in the oxidation of cellulosic substrates, which lead to rapid degradation of cellulose. This created more sites for the cellulases to bind and increased the hydrolysis rate. The surface oxidation of cellulose by Fenton reaction along with selective removal of lignin and partial hydrolysis of hemicellulose by the ligninolytic bacteria provided new insights into effective pretreatment strategies of lignocellulosic biomass (K. Zhang et al., 2018).

#### **III.1.2.4 Biological – Organosolv Pretreatment**

The organosolv process utilizes compounds like methanol, ethanol, butanol, n-butylamine, acetone, ethylene glycol, etc. to break the internal lignin and hemicellulose bonds and separate them. However, all the five articles discussed on biological-organosolv combined pretreatment (Table III-1) used ethanol for the pretreatment. Ethanol is preferred in an organosolv process as it is comparatively less toxic and due to its ease of recovery, thereby reducing the recurring costs of chemicals. In addition, all of them studied fungal pretreatment

followed by the organosolv process (ethanolysis) as fungal pretreatment helps to improve solvent accessibility, and thereby decreasing the severity of the organosolv process required. The 50-60% by volume ethanol-water solutions under high pressure of about 250 – 350 psi and high temperatures of about 180-200°C used in organosolv treatment effectively enhances delignification and produces easily hydrolyzable substrates. However, removal of the solvents is necessary because solvents could be inhibitory to the growth of organisms, enzymatic hydrolysis process, or the fermentation step. It has also been reported that organosolv pretreatment can better hydrolyze biomass with rather low lignin content. Therefore, a fungal pretreatment step before the organosolv process can help with reducing the lignin content and further increase solvent accessibility to biomass (Itoh et al., 2003; Muñoz et al., 2007). Results from Munoz et al. (2007), Kandhola et al. (2017), and Saad et al. (2008) who used white-rot fungi to treat wood showed that the majority of lignin degradation was due to biodegradation, which facilitated the action of chemicals further. Fissore et al. (2010) and Monroy et al. (2010) both studied the effect of brown rot fungi *Gloeophyllum trabeum* with organosolv process on *Pinus radiata* wood chips. The severity of the organosolv process based on the time and temperature of the process is described by the H factor. There were only slight severity differences in the organosolv process of these two studies; still, the fiber degradation obtained was quite varied. This shows that the same biomass from different sources undergoing similar pretreatment conditions could result in a varying amount of digestibility. The differences in the relative component contents of pretreated biomass could also arise as a result of different fungal species, culture conditions, culture time, and biomass (Yang et al., 2013). In the five cases discussed biological-organosolv combined pretreatment, it can be seen that fungal pretreatment facilitated the removal of lignin and hemicelluloses. This reduced the severity of the subsequent organosolv process required and produced a greater synergetic effect on cellulose digestibility by improving the solvent accessibility during the organosolv process. Another advantage of the organosolv process is that it presents low residual lignin and high glucan retention as can be observed from the five research articles discussed in Table III-1. This is because ethanol and the solubilized lignin act as scavengers for the free radicals formed during the organosolv cooking process, thereby reducing the extent of lignin condensation (Fissore et al., 2010).

#### **III.1.2.5 Biological – LHW/ HWE/ Autohydrolysis Pretreatment**

To avoid the use of chemicals for an environmentally friendly process, liquid hot water (LHW) pretreatment is preferred. This method is conducted at high temperatures of 120-260°C

at which water and acetyl groups in hemicelluloses act as acids and catalyze hemicellulose hydrolysis. These severe pretreatment conditions can result in organic acids accumulation and therefore create an acidic condition (Weil et al., 1998). To balance the acidic environment and therefore degradation of fermentable sugars, the severity of LHW pretreatment can be reduced by combining with biological pretreatment (Wan and Li, 2011). Wang et al. (2012) observed that while acid-soluble lignin (ASL) decreased with increasing temperature of LHW conditions, the acid-insoluble lignin (AIL) increased. The increase of AIL is attributed to condensation and precipitation of the lignin due to elevated temperatures whereas reduction in ASL is attributed to hot water liberating acids and thereby breaking ether linkages in biomass. Though LHW is credited for the high hemicelluloses solubilization, initial pretreatment with white-rot fungus is attributed for lowering the temperature needed to conduct LHW (Wang et al., 2012). Wan and Li (2011) observed that there was virtually no lignin removed by liquid hot water (LHW) pretreatment as observed generally in hydrothermal or thermochemical pretreatment. This is attributed to the condensation and precipitation of dissolved lignin and carbohydrate oligomers. However, Wan and Li (2011), suggest that LHW / hot water extraction (HWE) facilitates the fungal pretreatment in the subsequent step by initially reducing the recalcitrant of biomass. The difference between LHW and HWE as used by Wan and Li (2011) is that LHW is conducted at high temperature (170°C) and pressure (110 psi) in a sealed reactor while HWE is a method where the biomass is extracted with hot water at 85°C. The mechanism of LHW differs from HWE, in the sense that HWE helps to extract some hydrophilic compounds and lipophilic extractives that would impose a protective barrier to fungal degradation while LHW facilitates specifically lignin degradation for further fungal degradation. To illustrate the action of HWE, Wan and Li (2011) detected that water extractives in wheat straw partially contributed to recalcitrance to *C. subvermispora*, but an HWE pretreatment prior to fungal pretreatment partially removed water-soluble components of the biomass and significantly improved the sugar yield. It was also observed that different biomass reacts differently to both LHW and HWE depending on the varying amount of chemical bound components that cause recalcitrance in the crop residues (Wan and Li, 2011). Martin-Sampedro et al. (2015) combined endophytic/white rot fungi before and after a mild autohydrolysis as sequential pretreatment steps. A fungal pretreatment followed by mild alkaline pretreatment was more effective as it resulted in increased digestibility without masking the effect of fungal pretreatment. Irrespective of the order of pretreatment, the combined pretreatment enhanced the decrease in

Klason lignin and hemicellulose mainly in the form of xylose. As the first study on endophytic fungi in combined pretreatment, it is interesting to see the results of Martín-Sampedro et al. (2015) where the endophytic fungi performed comparative or enhanced lignin degradation as the white-rot studied for the same case. Even though the endophytic fungi *Pringsheimia smilacis* produced the highest degradation of lignin irrespective of the order of combined pretreatment steps, it did not produce higher saccharification rates. This study proved that endophytic fungi have the potential as primary degraders of lignocellulosic substrates and could be interesting to study further (Martín-Sampedro et al., 2015).

### **III.1.2.6 Biological – Steam Explosion Pretreatment**

Steam explosion pairs physical tearing and chemical high-temperature cooking of the biomass, which helps to degrade hemicelluloses and lignin while softening the cellulose (Li and Chen, 2014). To reduce the energy intensity of the steam explosion, it is often combined with biological pretreatment because of its easy integration to existing thermo-chemically treated biomass-to-ethanol processes (Keller et al., 2003). Vaidya and Singh (2012) compared the effect of brown-rot fungi and white-rot fungi on steam-exploded wood (SEW). SEW substrate was observed to be less recalcitrant than the raw biomass as the steam explosion caused some lignin degradation. There was more degradation in cellulose and hemicellulose of brown rot treated wood samples which caused higher weight losses in them compared to white rot treated wood samples. However, a steam explosion followed by white-rot fungus pretreatment was observed to work synergistically to enhance enzymatic digestion more than the brown rot fungus. Sawada et al. (1995) and Asada et al. (2011) conducted white rot fungal pretreatment followed by steam explosion at around 215°C for 5-6.5 minutes. Sawada observed that *P.chrysosporium* rapidly degraded 42% of lignin and gradually degraded holocellulose up to 17% of beech wood meal during an incubation time of 28 days. Later, the lignin degradation rate slowed down and the holocellulose degradation rapidly increased. Even though there was increase in the area of contact between holocellulose and the enzyme, it was not sufficient for enzymatic saccharification of the wood-meal. Therefore, a consecutive treatment with steam explosion was necessary to enhance saccharification. Above 210°C steam temperature, Sawada et al. (1995) observed that Klason lignin underwent condensation reactions with water-soluble material and methanol soluble lignin. Asada et al. (2011) also noticed that at higher temperatures of 214°C, recondensation of lignin occurs while the cellulose amount remained the same, as thermal degradation of cellulose is about 240°C. Therefore, at around 214°C or 20



atm pressure, there appears to be low Klason lignin which is desirable for the enzymatic or microbial conversion into sugars as Klason lignin decreases the susceptibility of enzyme and cellulose. On the other hand, Taniguchi et al. (2010) and Li and Chen (2014) conducted white-rot fungal pretreatment on steam-exploded crop residues for 1 minute with lower severity. Taniguchi et al. (2010) confirmed the structural changes in rice straw during the sole steam explosion and steam explosion- *P. ostreatus* combined pretreatment with SEM images. It was observed that the steam explosion solely did not effectively change the cellulose contents of lignocellulose while an increase in Klason lignin was observed due to partial condensation with other components. SEM micrographs helped to understand the impact of steam explosion and biological pretreatment on rice straw. The steam explosion only caused a partial cracking of the surface and slight destruction of the structure, while *P.ostreatus* hyphae growth on the surface and their invasion into the structural networks loosened the fibers and increased the surface area for enzymatic hydrolysis (Taniguchi et al., 2010). Li and Chen (2014) concluded similarly to Taniguchi et al. (2010) from results of pore size distribution, XRD analysis, and chemical composition of corn stalk by studying the effect of steam explosion, fungal treatment, and combined pretreatment strategy, which showed that steam explosion destroyed the rigid structure of the biomass and facilitated fungi penetration. *P. baumii*, being a white rot fungus selectively degrades lignin and enhances the effect of steam explosion. Therefore, from the five research articles discussed in Table III-1, it can be concluded that steam explosion is only advantageous because it does not degrade cellulose even at 30 atmospheric pressure and 235°C. Nevertheless, steam explosion needs another treatment in combination, preferably biological treatment with white rot fungi to offset the energy costs, to efficiently degrade lignin and increase the susceptibility of the biomass to enzymatic hydrolysis.

Therefore, it can be observed from Table III-1 that biological-alkaline and biological-organosolv combined pretreatment strategies helped to achieve higher fiber degradation. While pretreatment is the most important step in lignocellulosic biomass processing, an efficient enzymatic hydrolysis process is required to obtain an optimum yield of reducing sugar, which can then be used for various applications. The enzymatic hydrolysis process can be used to evaluate the effect of pretreatment more efficiently and therefore, the approach, their effect on different LCBs, and their pros and cons are discussed in Subchapter III.1.3.

### III.1.3 Evaluation of the combined pretreatment based on sugar yield

Biofuels are produced through either chemical reactions, bioconversion, or heat that help to break down the starches, sugars, and other molecules present in lignocellulosic biomass. Due to the association with complex polymers and crystalline state, cellulose is the key carbohydrate that needs to be hydrolyzed to release the hexose and pentose sugars it contains. Generally, enzymes or acids are used to catalyze the hydrolysis reaction. Enzymatic hydrolysis is better preferred due to the high specificity that can be achieved under milder conditions (pH around 4.8 and temperatures around 45 – 50°C). Moreover, enzymatic hydrolysis produces higher yields of glucose without introducing corrosion problems, which is favorable for subsequent processes. Three main steps occur during the enzymatic hydrolysis of cellulose: adsorption of cellulase enzymes to the surface of the cellulose, hydrolysis of cellulose to glucose, and desorption of cellulases. Cellulases consist of endoglucanases, exoglucanases, and  $\beta$ -glucosidase. The  $\beta$ -glucosidase activity is what helps to convert cellobiose and short-chain oligosaccharides into glucose. However, the commercially available cellulase enzymes normally show low  $\beta$ -glucosidase activity, causing incomplete cellobiose hydrolysis. Consequently, extra  $\beta$ -glucosidase enzymes are added to the hydrolysis mixture. Since the cellulase enzymes have to penetrate the cellulose structure for adsorption at the first step, pretreatment is necessary to remove hemicelluloses and lignin barriers and break the crystalline structure of cellulose. This would help to enhance the susceptibility to enzymatic hydrolysis (Chen, 2015; Gupta et al., 2016; Mesa et al., 2010; Ricardo Soccol et al., 2011). Therefore, the effectiveness of the pretreatment method could be analyzed by estimating the reducing sugar yield. The two widely used methods for determining reducing sugars are namely, the Nelson-Somogyi (NS) (used in 5 articles discussed in Table III-2) and 3,5-dinitrosalicylic acid (DNS) assays (used in 13 articles discussed in Table III-2). From Breuil and Saddler (1985) and Gusakov et al. (2011), it could be understood that the DNS method, although a very convenient method, overestimates the activity of enzymes and is susceptible to interference by various substances. On the other hand, though the Nelson and Somogyi copper method is more reliable and sensitive, it is not widely used because laboratories are reluctant to use the more toxic NS reagent which is also more sensitive to disturbing factors than DNS (Bailey et al., 1992). Another method (used in 2 articles discussed in Table III-2) for the determination of total sugars is the phenol-sulfuric acid method (DuBois et al., 1956), which although easier to use than many other available methods, poses multiple health hazards. The results of this method are

presented as glucose-equivalent concentrations and are not accurate for complex carbohydrates (Albalasmeh et al., 2013). These colorimetric methods can only be used for quantifying total reducing sugars (TRS) but not for the pentoses and hexoses separately. Instruments like high-performance anion-exchange chromatography (HPAEC) (used in 4 combined pretreatment studies discussed in Table III-2) and high-performance liquid chromatography (HPLC) (used in 16 combined pretreatment studies discussed in Table III-2) have been increasingly used for both quantitative and qualitative sugar analysis. Although they require high cost for analysis, they are regarded as the best methods (Chi et al., 2012).

The aim of the articles discussed in Table III-2 was to maximize the enzymatic digestibility by studying these combined pretreatment strategies. After the hydrolysis of the pretreated substrates, the substrates were either digested to produce biogas (as in the case of Zhao et al. (2017)) or fermented to produce ethanol. The sugar yields obtained from 35 articles using different combined biological and chemical/ physicochemical pretreated biomass are listed in Table III-2. The fold increase or decrease in sugar content after combined pretreatment as compared to sole pretreatment is calculated according to the formula given in Eq. III.1-1.

$$\text{Fold increase/ decrease} = \frac{\text{Sugar yield from combined pretreatment}}{\text{Sugar yield from sole pretreatment}} \quad (\text{Eq. III.1-1})$$

Biological- alkaline pretreatment is seen to be the most studied biological – chemical combined pretreatment strategies. In Table III-2, ten research articles are discussed for biological-alkaline combination, followed by eight and seven scientific publications for biological-acid and biological-oxidative combined pretreatment respectively. Five articles on the biological-organosolv process are also discussed in Table III-2. For biological-physicochemical combined pretreatment strategies, six papers on biological-steam explosion and three papers on biological- LHW/HWE are discussed.

**Table III-2:** Comparison of sugar yield of different microbial-chemical/physicochemical pretreatment strategies

Substrate	1st step	2 <sup>nd</sup> step	Hydrolysis method	Sugar yield	Fold increase	Reference	
<b>Biological – Alkaline Pretreatment</b>							
Wheat straw	<i>Ischnoderma benzoinum</i> 108 (28°C, 14 d)	2% (w/v) NaOH (115°C, 10 mins)	Cellulase (10 FPU/g) (40°C, 72 h, magnetically stirred) [Glucose by glucose oxidase method and RS by DNS]	388 mg/g (RS); 190 mg/g (G)	0.95 (RS) and 0.93 (G) fold less than sole alkali treatment*	Hatakka (1983)	
	<i>Pleurotus ostreatus</i> (28°C, 14 d)			308 mg/g (RS); 90 mg/g (G)			0.76 (RS) and 0.44 (G) fold less than sole alkali treatment*
	<i>Pycnoporus cinnabarinus</i> (28°C, 14 d)			343 mg/g (RS); 183 mg/g (G)			0.84 (RS) and 0.89 (G) fold less than sole alkali treatment*
<i>Pinus radiata</i>	<i>Gloeophyllum trabeum</i> (27°C, 28 d)	25% (w/w) NaOH (180°C, 5 h)	Celluclast (20 FPU/g) and $\beta$ -glucosidase (40 UI/g) (50°C,	77 $\pm$ 2% (G)	0.93-fold less than sole alkaline pretreatment*	Fissore et al. (2010)	

			24 h, 150 rpm) [HPLC]			
Cornstalks	<i>Irpex lacteus</i> CD2 (28°C, 15 d)	0.25 M NaOH (60°C, 2 h)	Cellulase (30 FPU/g) at (50°C, 48 h) [HPLC]	93.86% (glucan digestibility)	14% increase compared to sole alkaline pretreatment	Yu et al. (2010a)
Wheat straw ( <i>Triticum aestivum</i> )	<i>P. subvermispora</i> (28°C, 21 d)	0.1% NaOH (5% w/v) (50°C, 1 h, 165 rpm)	Cellulase (15 FPU/g) and xylanase (30 U/g) (50°C, 60 h, 165 rpm) [TRS by Somogyi, 1945; Glucose by Glucose TR kit]	357 ± 1 mg/g (G)	0.87-fold decrease than sole alkali pretreatment*	Salvachúa et al. (2011)
	<i>I. lacteus</i> (28°C, 21 d)			340 ± 2 mg/g (G)	0.83-fold decrease than sole alkali pretreatment*	
Cornstalks	<i>Irpex lacteus</i> (28°C, 15 d)	0.25 M NaOH (30°C, 2 h)	Cellulase (30 FPU/g of substrate) (50°C, 48 h) [DNS]	400.1 mg/g	1.31-fold increase than sole alkali pretreatment*	Zhong et al. (2011)
	<i>Echinodontium taxodii</i> (28°C, 15 d)			319.5 mg/g	1.05-fold increase than sole alkali pretreatment*	

<i>Populus tomentosa</i>	<i>Trametes velutina</i> D1014 (28°C, 56 d)	2.5 g NaClO <sub>3</sub> and 2 mL acetic acid (80°C, 1 h)	Cellulase (35 FPU/g) and β – glucosidase (37.5 CBU/g) (50°C, 72 h, 150 rpm) [HPAEC].	412.7 mg/g (84.77% cellulose conversion)	2.19-fold increase than sole fungal pretreatment*	Wang et al. (2013a)
<i>Populus tomentosa</i>	<i>Trametes velutina</i> D10149 (28°C, 28 d)	70% (v/v) ethanol aqueous solution containing 1% (w/v) NaOH (75°C, 3 h)	Cellulase (20 FPU/g) and β- glucosidase (30 CBU/g) (50°C, 144 h, 150 rpm) [HPAEC]	38.8% of cellulose conversion	4.8-fold increase than sole fungal pretreatment*	Yang et al. (2013)
Rice Straw	<i>Sphingobacterium</i> sp. LD-1 (30°C, 4 d)	4% NaOH + 6% Urea (-10°C, 4 h)	Cellulase (300 U/g) (47.5°C, 72 h, 100 rpm) [DNS]	RS: 9.25 mg/mL; G: 5.97 mg/mL	1.396- and 1.372-fold increase of RS and G respectively than sole alkaline pretreatment	Dai et al. (2015)
Maize Straw	1% w/v NaOH (room temperature, 48 h)	<i>T. harzianum</i> (30°C, 6 d)	No separate hydrolysis step [DNS]	0.52 mg/mL	0.96-fold decrease than sole alkali pretreatment*	Zhao et al. (2018)

		<i>Aspergillus</i> sp. (30°C, 6 d)		0.41 mg/mL	0.77-fold decrease than sole alkali pretreatment*	
		<i>T. harzianum</i> + <i>Aspergillus</i> sp. (30°C, 6 d)		0.66 mg/mL	1.23-fold increase than sole alkali pretreatment*	
		Enzyme T ( <i>T. harzianum</i> ) (50°C, 6 d)		0.63 mg/mL	1.18-fold increase than sole alkali pretreatment*	
		Enzyme A ( <i>Aspergillus</i> sp.) (50°C, 6 d)		0.74 mg/mL	1.38-fold increase than sole alkali pretreatment*	
		Enzyme T+A ( <i>T. harzianum</i> + <i>Aspergillus</i> sp.) (50°C, 6 d)		0.74 mg/mL	1.38-fold increase than sole alkali pretreatment*	
Rice straw	70 mL of ethanol-water solution	<i>Acinetobacter</i> sp. B-2 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C, 48 h, 110 rpm)	818.05 mg/g	1.32-fold increase than sole alkali pretreatment*	Si et al. (2019)

	(65:35, v/v) containing 0.5 wt % NaOH. 400 W of microwave irradiation for 10 min	<i>Bacillus</i> sp. B-3 (30°C, 2 d)		719.15 mg/g	1.16-fold increase than sole alkali pretreatment*	
		<i>Pandoraea</i> sp. B-6 (30°C, 2 d)		696.55 mg/g	1.12-fold increase than sole alkali pretreatment*	
		<i>Comamonas</i> sp. B-9 (30°C, 2 d)		611.77 mg/g	0.99-fold decrease than sole alkali pretreatment*	
<b>Biological – Acid Pretreatment</b>						
Water Hyacinth ( <i>Eichhornia crassipes</i> )	<i>Echinodontium Taxodii</i> (28°C, 10 d)	0.25% H <sub>2</sub> SO <sub>4</sub> (100°C, 1 h)	Cellulase (30 FPU/g) (50°C, 48 h) [DNS]	366.0 mg/g dry matter	1.13-fold increase than acid treatment alone*	Ma et al. (2010)
<i>Glycyrrhiza uralensis</i> Fisch. Ex DC	2.5% H <sub>2</sub> SO <sub>4</sub> (100°C, 2.5 h)	<i>Phanerochaete chrysosporium</i> (28°C, 21 d)	Cellulase (30 FPU/g) (50°C, 48 h) [DNS]	192.07 mg/g	1.09-fold increase than acid pretreatment alone*	Gui et al. (2013)
<i>Populus tomentosa</i>	<i>Trametes velutina</i> D1014 (28°C, 56 d)	1% H <sub>2</sub> SO <sub>4</sub> (140°C, 2 h)	Cellulase (35 FPU/g) and β–glucosidase (37.5	326.73 mg/g (65.58% cellulose conversion)	1.74-fold increase than fungal pretreatment alone*	Wang et al. (2013a)



			CBU/g) (50°C, 72 h, 150 rpm) [HPAEC].			
Corn Leaf	<i>Trametes</i> sp. 44 (PS 8, 200mL of air/ min, 13 d)	4% v/v H <sub>2</sub> SO <sub>4</sub> (121°C, 24 h, 1.5 atm)	Xylanase (63 AU) and Cellulase (5.44 AU) (50°C, 48 h) [RS by DNS; simple sugars by HPLC]	12.2 g/L	9% increase	Ángeles Ramírez et al. (2014)
Olive tree pruning	<i>Ulocladium</i> sp. (23°C, 28 d)	0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm); 0.5% (w/w) H <sub>2</sub> SO <sub>4</sub> (130°C, 1 h)	Cellulolytic complex (15 FPU/g) and β-glucosidase (15 IU/g) (50°C, 168 h, 120 rpm) [HPLC]	136 mg/g	0.99-fold decrease compared to fungi pretreatment with alkali extraction*	Martín-Sampedro et al. (2017)
	<i>Hormonema</i> sp. (23°C, 28 d)			130 mg/g	0.94-fold decrease compared to fungi pretreatment with alkali extraction*	
	<i>Trametes</i> sp. (23°C, 28 d)			131 mg/g	0.95-fold decrease compared to fungi pretreatment with alkali extraction*	

Rice straw	0.5% H <sub>2</sub> SO <sub>4</sub> (121°C, 40 min)	<i>Cupriavidus basilensis</i> B-8 (30°C, 3 d)	Cellulase (50°C, 72 h, 120 rpm) [DNS]	442 mg/g	1.7-fold increase than sole acid pretreatment*	Yan et al. (2017)
Olive tree biomass	2% (w/v) H <sub>2</sub> SO <sub>4</sub> (130°C, 1.5 h)	<i>Irpex lacteus</i> (Fr.238 617/93) (30°C, 28 d)	Cellulase (15 FPU/ g), β-glucosidase (15 IU/ g) and xylanase (30 U/ g) (50°C, 72 h, 150 rpm) [HPLC]	5.1 ± 0.5 g/L (G)	0.69-fold decrease than acid pretreatment alone*	Martínez-Patiño et al. (2018)
Olive tree biomass	<i>Irpex lacteus</i> (Fr.238 617/93) (30°C, 28 d)	2% (w/v) H <sub>2</sub> SO <sub>4</sub> (130°C, 1.5 h)	Cellulase (15 FPU/g), β-glucosidase (15 IU/g) and xylanase (30 U/g) (50°C, 72 h, 150 rpm) [HPLC]	9.9 ± 0.1 g/L (G)	1.34-fold increase than acid pretreatment alone*	Martínez-Patiño et al. (2018)
Rice straw	70 mL of ethanol-water solution	<i>Acinetobacter</i> sp. B-2 (30°C, 2 d)	Cellulase (12 FPU/g)	662.64 mg/g	1.21-fold increase than acid pretreatment alone*	Si et al. (2019)

	(65:35, v/v) containing 0.5 wt % HCl. 400W of microwave irradiation for 10 min	<i>Bacillus</i> sp. B-3 (30°C, 2 d)	(50°C, 48h, 110 rpm)	703.61 mg/g	1.29-fold increase than acid pretreatment alone*	
		<i>Pandoraea</i> sp. B-6 (30°C, 2 d)		770.02 mg/g	1.41-fold increase than acid pretreatment alone*	
		<i>Comamonas</i> sp. B-9 (30°C, 2 d)		599.06 mg/ g	1.10-fold increase than acid pretreatment alone*	
<b>Biological - Oxidative Pretreatment</b>						
Rice Hull	H <sub>2</sub> O <sub>2</sub> (2%, 48 h)	<i>Pleurotus ostreatus</i> (28°C, 18 d)	Cellulase (15 FPU/mg) (45°C, 48 h, 150 rpm) [TS by phenol–sulfuric acid (Dubois et al., 1956) and G by glucose oxidase–peroxidase]	39.8% (Total Sugars) 49.6% (G)	5.8 times (Total sugars) and 6.5 times (G) more than sole fungal pretreatment for 18 d	Yu et al. (2009)

Corn Straw	<i>Echinodontium taxodii</i> (25°C, 30 d)	0.0016% NaOH and 3% H <sub>2</sub> O <sub>2</sub> (room temperature, 16 h)	2 mg/mL Cellulase (100 FPU/g) (50°C, 48 h) [DNS]	57.52%	1.43-fold increase than sole A/O pretreatment at 8 mg/ mL cellulase concentration*	Yu et al. (2010b)
<i>Populus tomentosa</i>	<i>Trametes orientalis</i> (28°C, 28 d)	FeCl <sub>3</sub> (180°C, 30 mins)	Cellulase (30 FPU/g) and β - glucosidase (37.5 IU/g) (50°C, 96 h, 150 rpm) [DNS]	84.5%	1.4-fold increase than sole FeCl <sub>3</sub> treatment	Wang et al. (2013b)
	<i>Fomitopsis palustris</i> (28°C, 28 d)			95.4%	1.6-fold increase than sole FeCl <sub>3</sub> treatment	
Wheat straw	microbubble mediated ozonolysis (pH 3, 8.87 mg/L, 24 h)	<i>Pseudomonas putida</i> KT2440 (30°C, 48 h)	[DNS]	1.1 ± 0.09 mg/mL	1.83-fold increase than sole ozonation pretreatment*	
Hemp chips	<i>Pleurotus eryngii</i> (28°C, 21 d)	3% NaOH and 3% (v/v) H <sub>2</sub> O <sub>2</sub> (40°C, 24 h)	Cellulase (30 FPU/g) (50°C, 48h) [DNS]	372 mg/g	1.29-fold increase than sole chemical treatment*	Xie et al. (2017)

Rice Straw	0.02 M FeCl <sub>3</sub> , 1.5 M H <sub>2</sub> O <sub>2</sub> (25°C, 2 h)	<i>Cupriavidus basilensis</i> B-8 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C, 72 h, 120 rpm) [DNS]	462.74 mg/g	1.54-fold increase than sole Fenton treatment*	Zhang et al. (2018)
	<i>Cupriavidus basilensis</i> B-8 (30°C, 2 d)	0.02 M FeCl <sub>3</sub> , 1.5 M H <sub>2</sub> O <sub>2</sub> (25°C, 2 h)		326.80 mg/g	1.09-fold increase than sole Fenton treatment*	
Rice straw	70 mL of ethanol–water solution (65:35, v/v) containing 0.5 wt % FeCl <sub>3</sub> . 400W of microwave irradiation for 10 min	<i>Acinetobacter</i> sp. B-2 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C, 48h, 110 rpm)	361.13 mg/g	2.02-fold increase than sole FeCl <sub>3</sub> treatment*	Si et al. (2019)
		<i>Bacillus</i> sp. B-3 (30°C, 2 d)		297.65 mg/g	1.66-fold increase than sole FeCl <sub>3</sub> treatment*	
		<i>Pandoraea</i> sp. B-6 (30°C, 2 d)		287.77 mg/g	1.61-fold increase than sole FeCl <sub>3</sub> treatment*	
		<i>Comamonas</i> sp. B-9 (30°C, 2 d)		259.56 mg/g	1.45-fold increase than sole FeCl <sub>3</sub> treatment*	
<b>Biological – Organosolv Pretreatment</b>						

Sapwood of beech ( <i>Fagus crenata</i> )	<i>C. subvermispora</i> FP90031 (28°C, 28 d)	60% (v/v) ethanol solution (180°C, 2 h)	Meicellase (224 FPU/g) and $\beta$ - glucosidase activity (264 IU/g) (45°C, 96 h, 170 rpm) [Somogyi- Nelson]	0.454 g/g (G)	1.03-fold increase than sole organosolv pretreatment*	Itoh et al. (2003)
<i>Pinus radiata</i> woods chips	<i>Ceriporiopsis</i> <i>subvermispora</i> (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 11,360); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)	Cellulase (20 FPU/g glucan) and $\beta$ - glucosidase (40 CBU/g glucan) (50°C, 72 h, 150 rpm) [HPLC]	92.1 $\pm$ 0.5% Glucan: 100% glucan-to- glucose conversion	1.13-fold increase than sole organosolv pretreatment*	Muñoz et al. (2007)
<i>Acacia dealbata</i> woods chips	<i>Ganoderma australe</i> (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 10,920); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline	Cellulase (20 FPU/g glucan) and $\beta$ - glucosidase (40 CBU/g glucan) (50°C,	93.8 $\pm$ 0.4% glucan; 100% glucan-to- glucose conversion	1.03-fold increase than sole organosolv pretreatment*	Muñoz et al. (2007)

		wash: 1% NaOH (75°C, 1 h)	12 h, 150 rpm) [HPLC]			
<i>Pinus radiata</i>	<i>Gloeophyllum trabeum</i> (27°C, 28 d)	60% ethanol in water solvent (200°C, 1 h)	Celluclast (20 FPU/ g pulp) and $\beta$ -glucosidase (40 UI/ g pulp) (50 °C, 24 h, 150 rpm) [HPLC]	82.4% (G)	1.10-fold increase than sole organosolv pretreatment*	Fissore et al. (2010)
Japanese cedar ( <i>Cryptomeria japonica</i> )	<i>Phellinus</i> sp. SKM2102 (28°C, 56 d)	Ethanol/ lactic acid/ water (40:10:50, w/w) (190°C, 30 mins)	Meicellase (224 FPU/g) and $\beta$ -glucosidase (264 IU/g) (45°C, 96 h, 170 rpm) [Somogyi-Nelson method]	442 mg/g (63.5%)	7 times increased	Baba et al. (2011)
	<i>C. subvermispora</i> FP- 90031-sp (28°C, 56 d)	Ethanol/ lactic acid/ water (40:10:50, w/w) (200°C, 1 h)		52%	7.32-fold increase	

Pinewood chips	<i>T. versicolor</i> (28°C, 15 d)	65% ethanol in water solvent with 1% H <sub>2</sub> SO <sub>4</sub> (v/v) (170°C, 1 h)	Accellerase® 1500 enzyme (15 FPU/g glucan) (50 °C, 72 h) [HPLC]	32.0 ± 0.9% Glucan content with 100% glucan conversion	1.09-fold increase than sole organosolv pretreatment*	Kandhola et al. (2017)
<b>Biological – LHW/ HWE/ Autohydrolysis Pretreatment</b>						
Soybean	Liquid Hot water (170°C, 3 mins, 400 rpm, 110 psi, solid to liquid ratio of 1:10)	<i>Ceriporiopsis subvermispota</i> (28°C, 18 d)	Spezyme CP (10 FPU/g) (50°C, 72 h, 130 rpm) [HPLC]	64.29% (G)	1.51-fold increase than sole LHW pretreatment*	Wan and Li (2011)
Corn stover				53% (G)	1.54-fold increase than sole LHW pretreatment*	
Wheat straw	Hot water extraction (HWE) (85°C, 10 mins,	<i>Ceriporiopsis subvermispota</i> (28°C, 18 d)	Spezyme CP (10 FPU/g) (50°C, 72	43.69% (G)	2.26-fold increase than sole HWE pretreatment*	Wan and Li (2011)



Corn stover	solid to liquid ratio of 1:20).		h, 130 rpm) [HPLC]	55.19% (G)	2.44-fold increase than sole HWE pretreatment*	
Soybean				35.24% (G)	1.05-fold increase than sole HWE pretreatment*	
<i>Populus tomentosa</i>	<i>Lenzites betulina</i> C5617 (28°C, 28 d)	Liquid hot water (LHW) (200°C, 30 mins, 10% w/v of dry matter mixture)	Cellulase (35 FPU/g) (50°C, 96 h, 150 rpm) [HPAEC]	60.29% (G)	2.66-fold increase than sole LHW pretreatment	Wang et al. (2012)
	<i>Trametes ochracea</i> C6888 (28°C, 28 d)			58.79% (G)	1.12-fold increase than sole LHW pretreatment*	
<i>Eucalyptus globulus</i>	Autohydrolysis (135°C, 30 mins, Liquid to solid ratio of 6:1)	<i>Ulocladium</i> sp. (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)	Celluclast (15 FPU/g) and $\beta$ -glucosidase (15 U/g) (50°C, 72 h, 120 rpm) [HPLC]	9.03 g/L (G) and 11.22 g/L (Total Sugars)	3.29 (G) and 3.34 (Total Sugars) fold increase than sole autohydrolysis process*	Martín-Sampedro et al. (2015)
		<i>Hormonema</i> sp (23°C, 28 d); 0.1%		8.17 g/L (G) and 10.19 g/L (Total Sugars)	2.98 (G) and 3.03 (Total Sugars) fold increase than sole	

		NaOH (5% w/w) at (50°C, 1 h, 165 rpm)			autohydrolysis process*	
		<i>Trametes</i> sp.(23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		6.28 g/L (G) and 8.04 g/L (Total Sugars)	2.29 (G) and 2.39 (Total Sugars) fold increase than sole autohydrolysis process*	
		<i>Pringsheimia smilacis</i> (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		5.55 g/L (G) and 7.01 g/L (Total Sugars)	2.02 (G) and 2.08 (Total Sugars) fold increase than sole autohydrolysis process*	
<i>Eucalyptus globulus</i>	<i>Ulocladium</i> sp. (23°C, 28 d)	0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm); Autohydrolysis (135°C, 30 mins, Liquid to solid ratio of 6:1)	Celluclast (15 FPU/g) and $\beta$ -glucosidase (15 U/g) (50°C, 72 h, 120 rpm) [HPLC]	8.73 g/L (G) and 11.26 g/L (Total Sugars)	3.18 (G) and 3.35 (Total Sugars) fold increase than sole autohydrolysis process*	Martín-Sampedro et al. (2015)
	<i>Hormonema</i> sp. (23°C, 28 d)			8.35 g/L (G) and 10.65 g/L (Total Sugars)	3.04 (G) and 3.17 (Total Sugars) fold increase than sole	

					autohydrolysis process*	
	<i>Trametes</i> sp. (23°C, 28 d)			4.93 g/L (G) and 6.02 g/L (Total Sugars)	1.80 (G) and 1.79 (Total Sugars) fold increase than sole autohydrolysis process*	
	<i>Pringsheimia smilacis</i> (23°C, 28 d)			5.58 g/L (G) and 7.01 g/L (Total Sugars)	2.03 (G) and 2.08 (Total Sugars) fold increase than sole autohydrolysis process*	
<b>Biological – Steam Explosion Pretreatment</b>						
Beech wood	<i>Phanerochaete chrysosporium</i> (37°C, 28 d)	Steam explosion (210°C, 10 mins)	Cellulase (37°C, 100 h) [Somogyi-Nelson method]	76% (saccharification)	1.13-fold increase than sole steam explosion pretreatment*	Sawada et al. (1995)
Rice straw	<i>Pleurotus ostreatus</i> (25°C, 23 d)	AFEX (Liquid ammonia loading 1:1,	Cellulase (15 FPU/g glucan), $\beta$ glucosidase (64	92% glucan and 55% xylan conversion	1.12-fold increase in glucan	Balan et al. (2008)

		80% moisture) (100°C, 5 mins)	<i>p</i> NPGU/g glucan) and xylanase (10% of cellulose protein) (50°C, 168 h, 90 rpm) [HPLC]		conversion than untreated biomass*	
Rice straw	Steam explosion (1.5 MPa, 1 min)	<i>Pleurotus ostreatus</i> ATCC 66376 (25°C, 36 d)	Cellulase (40°C, 48 h) [glucose oxidase– peroxidase kit]	33% (G)	1.8-fold more conversion than sole fungal pretreatment*	Taniguchi et al. (2010)
Sawtooth oak (90%), corn and bran (10%)	<i>Lentinula edodes</i> (120 d)	Steam explosion (214°C, 5 mins, 20 atm/ 2.03 MPa)	Enzyme (45°C, 48 h, 140 strokes/min) [TRS by Somogyi– Nelson method; Glucose by mutarotase GOD]	62 mg/g (G)	1.94-fold increase than fungal pretreatment alone*	Asada et al. (2011)
<i>Pinus radiata</i>	Steam explosion (235°C, 1 min)	<i>Coniophora puteana</i> ( <i>Schumach.</i> ) <i>P. Karst</i> (26°C, 42 d)	Celluclast (20 FPU/g) β- glucosidase (25	5.86 g/L (G)	1.13-fold increase than sole steam- exploded wood*	Vaidya and Singh (2012)

		<i>Antrodia xantha</i> (Fr.) <i>Ryvarden</i> (26°C, 42 d)	IU/g) (50°C, 24 h, 180 rpm) [YSI-2700D glucose analyzer]	6.38 g/L (G)	1.23-fold increase than sole steam-exploded wood*	
		<i>Oligoporus placenta</i> (Fries) Gilb and <i>Ryvarden</i> (26°C, 42 d)		6.28 g/L (G)	1.21-fold increase than sole steam-exploded wood*	
		<i>Trametes versicolor</i> (L.) Lloyd (26°C, 42 d)		6.40 g/L (G)	1.23-fold increase than sole steam-exploded wood*	
Corn stalk	Steam explosion (1.7 MPa, 1min)	<i>Phellinus baumii</i> (28°C, 21 d)	Cellulase (20 FPU/g) (50°C, 48 h, 130 rpm) [HPLC]	313.31 mg/g (G)	1.32 times increase than sole steam explosion	Li and Chen (2014)

\*Calculated by the authors according to Eq. III.1-1 with data in the literature

(G): Glucose; (RS): Reducing sugars

### III.1.3.1 Biological – Alkaline Pretreatment

In most cases of biological-alkaline combined pretreatment, fungal pretreatment was conducted before alkali pretreatment because it has been reported to enhance cellulose digestibility and also reduce the production of inhibitors that are toxic for subsequent fermentation (Salvachúa et al., 2011). Of all biological-chemical combined pretreatment studied, many of the biological-alkaline pretreatment studies produced lower reducing sugar yield compared to the single pretreatment step. Even though Hatakka (1983), Fissore et al. (2010) and Salvachúa et al. (2011) studied fungal pretreatment followed by alkali pretreatment similar to that of Zhong et al. (2011), Yang et al. (2013), Wang et al. (2013a), Yu et al. (2010a) and Dai et al. (2015) their results are not in correspondence. Indeed Hatakka (1983), and Fissore et al. (2010) conducted alkaline delignification at much higher temperatures (115°C and 180°C respectively) and yet obtained lower reducing sugar yield from combined pretreatment, unlike the rest who conducted alkaline pretreatment in the range of 25°C - 80°C. This was presumed to be because of a lower degree of polymerization of cellulose chains in fungal treated wood in the case of Fissore et al. (2010) which resulted in higher amounts of carbohydrates solubilized than lignin and therefore, the effect of strong alkali treatment being masked off by the effect of fungi. The other plausible reason for the low reducing sugar yield in the study by Hatakka (1983) could be the low efficiency of the cellulase enzyme used in the hydrolysis step. Therefore, the saccharification efficiency is dependent on both the pretreatment and the enzyme efficiency. Generally, alkali treatment helps in the removal of the hydrophobic restriction of lignin and alters the lignin-hemicellulose network while leaving the high crystalline cellulose unchanged (Si et al., 2019). To enhance the saccharification, Si et al. (2019) combined alkali pretreatment with bacteria. Saccharolytic bacterium *Acinetobacter* sp. B-2 significantly enhanced the sugar yield in the combined pretreatment with alkali. In another study by Zhao et al. (2018) where pretreatment of maize straw was carried out with *T. harzianum*, with the enzyme of *T. harzianum* and pretreatment with NaOH followed by *T. harzianum*, the reducing sugar concentrations were 1.20 mg/ mL, 2.20 mg/ mL and 0.19 mg/ mL. The decrease in the reducing sugar yield in the combined pretreatment method was presumed to be due to the washing away of soluble material dissolved during alkali treatment and the greater ability of the fungi to consume than to produce reducing sugar (Zhao et al., 2018). Hence, pretreatment with enzymes rather than microorganisms themselves was more effective.

### III.1.3.2 Biological – Acid Pretreatment

The dilute acid pretreatment is primarily a hydrolytic process that causes solubilization of hemicellulosic sugar and therefore, a fungal pretreatment step is required first to degrade the lignin in the biomass and make cellulase enzyme accessibility to cellulose easier (Shirkavand et al., 2016). Many studies on only acid pretreatment have reported an initial increase and later a drop in TRS yield with an increase in acid concentration, reaction time, and temperature. This is presumed to be due to the formation of inhibitory products. Therefore, mild acidic conditions are preferred to obtain a high yield of TRS (Kootstra et al., 2009; Rajan and Carrier, 2014; Timung et al., 2016). To make mild acidic pretreatment more effective, combining with fungal pretreatment is seen to improve the sugar yields from 1.09-1.74 fold (Gui et al., 2013; Ma et al., 2010; Wang et al., 2013a). Martínez-Patiño et al. (2018) compared sequential chemical and biological pretreatment and vice versa for enzymatic hydrolysis of olive tree biomass. The best combination to significantly increase the glucose concentration was biological pretreatment followed by mild acid pretreatment. Moreover, acid treatment as a first step could produce some inhibitors which may hinder the fungal pretreatment efficiency (Martínez-Patiño et al., 2018). Ramirez et al. (2014) performed fungal pretreatment followed by acid hydrolysis and enzymatic hydrolysis of corn leaf and obtained a 9% increase in the reducing sugar yield as compared to sole pretreatment. This increase could be attributed to the multiple-step hydrolysis process, which helped to obtain higher saccharification of the pretreated biomass while making this process more complex to carry out. In the study of Martín-Sampedro et al. (2017), although the endophytic fungi could enhance sugar yield, there was no significant increase in sugar yield obtained during combined pretreatment. This was attributed to the fungal effectiveness hindered by the high water-soluble extractives content of olive tree pruning. The authors concluded that better pretreatment strategies need to be explored to commercially valorize olive tree pruning biomass. Yan et al. (2017) and Si et al. (2019) observed the complementary effect of acid hydrolysis which mainly deconstructs the cellulose and hemicelluloses along with ligninolytic bacterium, improved the digestibility significantly. Their results served new insights into bacteria-acid synergy for the pretreatment of lignocellulosic biomass. Its potential is interesting to explore further as the use of bacteria reduces the pretreatment time and the associated costs (Si et al., 2019; Yan et al., 2017).

### III.1.3.3 Biological - Oxidative Pretreatment

The combination of powerful oxidant ( $H_2O_2$ ) followed by white-rot fungi (*Pleurotus ostreatus*) to pretreat rice hull by Yu et al. (2009) showed enhanced net yields of sugar, while effectively reducing the pretreatment time from 60 days for sole fungal pretreatment to 18 days for combined pretreatment. The reduction in the carbohydrates' loss was also minimal as can be seen from Table III-1. The structural changes observed from SEM results are mainly attributed to easy penetration of fungal hyphae into the rice hull structure chemically degraded by  $H_2O_2$  first and thereby increased production of sugar almost 6 times than that of sole fungal pretreatment (Yu et al., 2009). In the study of white-rot/ brown-rot fungus followed by mild oxidizing agent ( $FeCl_3$ ) treatment of poplar wood (Wang et al., 2013b), an increase in temperature during oxidant treatment led to a further increase in sugar yields. From Table III-1, it could be observed that there is no delignification due to both fungal or oxidant treatment while a higher hemicelluloses degradation was observed in the combined treatment. Yet, the synergy of combined treatment increased the internal surface area and porosity, leading to a decrease in the unproductive binding of the enzyme with lignin. Wang et al. (2013b) presume that an alteration in the structure of lignin such as a change in the content of hydrophilic phenolic hydroxyl groups could have led to a reduction in the enzyme's irreversible adsorption. These factors thereby increased the enzyme accessibility to cellulose (Wang et al., 2013b). To decrease the power and energy demands during the oxidative process, Yu et al. (2010b) and Xie et al. (2017) used oxidative pretreatment under mild alkaline conditions but this did not enhance the enzymatic hydrolysis process. So, by using white-rot fungal pretreatment along with alkaline-oxidative pretreatment, a higher sugar yield at lower enzyme concentration was achieved, thereby reducing the cost of an enzymatic hydrolysis step (Yu et al., 2010b). From the cellulase adsorption study, Yu et al. (2010b) concluded that the increased yield of reducing sugar was obtained due to a decrease in unproductive adsorption during biological pretreatment. Another important conclusion (based on the results not shown here) by Yu et al. (2010b) was that even though, *T.versicolor* showed the highest lignin-degrading ability, it also produced high cellulose loss which ensued in lower reducing sugar yields. This highlights the need to minimize cellulose loss. An efficient way to specifically target the lignin molecule is by ozonation. Ozone is highly reactive with compounds containing double bonds and high electron densities such as lignin (García-Cubero et al., 2009). These reactions follow the Criegee mechanism and no by-products are formed during the degradation process (Mulakhudair et al.,



2017). The study by Mulakhudair et al. (2017) showed that ozonation for 24 hours reduced the biological pretreatment time by 50% but more importantly, a substantial increase in microbial biomass. A drastic increase in glucose concentration of 323% was observed when the ozonation time was increased from 2 h to 24 h. By using microbubble-mediated ozonolysis, there was a significant improvement in dosage efficiency due to the high surface area to volume ratio. Nonetheless, the high cost of the ozonation process makes it an expensive pretreatment method. To lower the cost of the pretreatment process, Zhang et al. (2018) designed a biomimetic system. In nature, fungi and bacteria are in a symbiotic relationship to utilize lignocellulose. The fungi modify the recalcitrant cell wall barrier using an oxidative step and release small molecular compounds which are further degraded by bacteria (Alper and Stephanopoulos, 2009). Therefore, Zhang et al. (2018) utilized low-cost Fenton catalyst ( $\text{Fe}^{3+}$  and  $\text{H}_2\text{O}_2$ ) to stimulate fungal invasion of plant tissue, combined with *Cupriavidus basilensis* B-8 that helped enhance the enzymatic hydrolysis process. On the other hand, the sequential treatment with bacteria followed by the Fenton catalysts had a lower reducing sugar yield (K. Zhang et al., 2018). Si et al. (2019) also studied the combination of metal salt  $\text{FeCl}_3$  with various saccharolytic and ligninolytic bacteria. Though there was a significant increase in the sugar yield as compared to that of sole  $\text{FeCl}_3$  treatment, the reducing sugar yield was still lower due to negligible change in the chemical composition of rice straw (Si et al., 2019).

#### **III.1.3.4 Biological – Organosolv Pretreatment**

Wood being one of the most recalcitrant biomasses needs efficient pretreatment methods to be developed to improve its saccharification. Combined pretreatment using fungi followed by the organosolv process is an environmentally benign treatment for wood (Baba et al., 2011). Baba et al. (2011), Fissore et al. (2010), Itoh et al. (2003), Kandhola et al. (2017), and Muñoz et al. (2007) have all reported having an increase in the sugar yield by both brown rot and white rot fungi when combined with ethanolsis.

#### **III.1.3.5 Biological – LHW/ HWE/ Autohydrolysis Pretreatment**

It has been established by many researchers (Liu, 2010; Mosier, 2005; Zeng et al., 2007) that at elevated temperature and pressure, LHW pretreatment is comparatively an environment-friendly pretreatment as it has no sludge generation and limited corrosion problems. At temperatures around 200°C, the water and acetyl groups inside hemicelluloses act as acids that catalyze the hemicellulose hydrolysis to mainly xylose. The synergy of biological-LHW

combined pretreatment is promising according to the results of Wang et al. (2012), which helps to lower the severity of LHW pretreatment while enhancing biological pretreatment efficiency. The high glucose yield obtained when LHW was carried out at 200°C was attributed to the hemicellulose loss, which facilitates the enzymatic hydrolysis of poplar wood. However, the researchers saw a decrease in the ratio between glucose yield of combined pretreatment and that of sole LHW as the temperature of LHW was increased from 140 to 200°C. This shows that the LHW treatment has higher efficiency at a higher temperature in both combined or sole pretreatment (i.e. 200°C), but the combination with fungal pretreatment triggered a better improvement of the yield at low temperature (Wang et al., 2012). Besides, the more severe the pretreatment conditions are, the more effective is the LHW pretreatment but it also results in the accumulation of inhibitory compounds like hydroxymethylfurfural (HMF), furfural, formic acid, levulinic acid (Weil et al., 1998). Therefore, it is suggested to carry out LHW at less severe conditions and then follow with other pretreatment methods. Wan and Li (2011) studied LHW/HWE along with *Ceriporiopsis subvermispora* for different biomasses such as soybean, corn stover, and wheat straw. The synergistic effect of the combined LHW and fungal pretreatment process was significant for soybean straw whereas not so much for corn stover. The glucose yield obtained in the combined study was higher for soybean and not for corn stover when compared to sole fungal pretreatment. In the combined hot water extraction (HWE) and fungal pretreatment, there was an almost 2-fold increase in the glucose yield compared to untreated/fungal-/ HWE- pretreated wheat straw, while soybean showed no increase in yield in the combined pretreatment compared to individual pretreatment step. On the other hand, combined pretreated corn stover showed a drastic increase in glucose yield as compared to HWE pretreated biomass but a slight decrease compared to fungal pretreated biomass. Thus, their research indicated how different biomasses show different pretreatment efficiency to the same combined pretreatment method (Wan and Li, 2011). Martín-Sampedro et al. (2015) used highly specific endophytic fungi with a mild autohydrolysis process to enhance saccharification of *Eucalyptus globulus* wood. The endophytic fungi produced higher saccharification than white-rot fungi, while the mild autohydrolysis helped to boost the fungal effect. It was also established from the results that the highest lignin-degrading fungi (*P. smilacis*) did not produce the greatest saccharification, indicating that the extent of lignin removal is not always correlated with the enhancement of saccharification yields (Martín-Sampedro et al., 2015).

### III.1.3.6 Biological – Steam Explosion Pretreatment

All studies done so far on biological-steam explosion combined pretreatment produced a significant increase in the net sugar yields (Asada et al., 2011; Balan et al., 2008; Li and Chen, 2014; Sawada et al., 1995; Taniguchi et al., 2010; Vaidya and Singh, 2012). Sawada et al. (1995) observed that a sole fungal treatment was not sufficient to increase the enzymatic saccharification of beech wood even though a large amount of lignin had degraded paving the way for increasing contact between enzyme and holocellulose. Consecutive treatment with steam explosion helped to enhance the saccharification. When either steaming time or steam temperature was increased with the other constant, the saccharification increased up to its maximum (82% at a steam temperature of 215°C and steaming time of 6.5 mins) and then decreased with further increase. High steam temperature or longer steaming time caused depolymerized lignin to combine with the holocellulose which in turn led to holocellulose being unsusceptible to the enzyme (Sawada et al., 1995). Asada et al. (2011) carried out a steam explosion on spent shiitake mushroom media (*Lentinula edodes* mushroom grown on a media containing corn and bran for four months). After the harvest of the fruiting bodies, steam explosion pretreatment proved useful for the effective utilization of the spent medium for biofuel production (Asada et al., 2011). Balan et al. (2008) also studied spent oyster mushroom rice straw media to utilize as a potential substrate for biofuel production. The fungal pretreatment helped to reduce the severity of the subsequent AFEX treatment by improving accessibility to chemicals and enzymes and lead to a 15% increase in glucan (Balan et al., 2008). The opposite sequence of pretreatment (steam explosion followed by fungal pretreatment) was also observed to be effective for the conversion of biomass into sugars. Li and Chen (2014), Taniguchi et al. (2010), and Vaidya and Singh (2012) ascertained the effectiveness to the lignin-carbohydrate complex of biomass being destroyed by the steam explosion which further facilitated the fungal treatment.

From all the combined pretreatment methods discussed in this chapter, we can conclude that the order of biological and chemical methods in successive pretreatments should be chosen based on the mode of action of chemical/physicochemical pretreatment. Biological pretreatment combined with oxidative / ethanolysis seems to be the most effective biological-chemical combined pretreatment while mild autohydrolysis followed by endophytic fungi seems to be the most effective biological-physicochemical combined pretreatment, based on the fold increase of sugar yield compared to a sole pretreatment strategy. The hydrolysis rate

depends on the ratio of total enzyme ratio to the amount of substrate added. For the quantification of the pretreatment efficiencies, most of the studies used cellulase loading of more than 15 FPU/g as high enzyme doses are required to release sugars from naturally recalcitrant biomasses (Yang et al., 2011). Hydrolysis of lignocellulosic varies with cellulases absorption and efficacy, hemicelluloses and lignin removal, and accessible surface area (Karimi and Taherzadeh, 2016). Therefore, each research group uses a different concentration of enzymes and different residence times to alter the rate of biomass deconstruction into fermentable sugars. According to Breuil and Saddler (1985), the enzyme concentration does not proportionally affect the reducing sugar values obtained from the hydrolysis step. Nevertheless, pretreatment could alter the structure and composition differently for the same biomass from different regions, which could result in different reducing sugar yields. It is noteworthy that the composition of each lignocellulosic biomass could also vary with geographical location and seasons. These non-standardized conditions make it difficult for direct comparison of the sugar yields from different combined pretreatment strategies. In the future, improved analytical methods to determine the enzyme-substrate interaction could help to better optimize the hydrolysis step for each biomass specifically.

#### **III.1.4 Conclusion**

The correlation between the biomass properties and its degradability remains unclear, even though many researchers have evaluated the effect of pretreatment. Based on the biomass and the downstream process, the appropriate pretreatment steps need to be chosen. It is also important to establish the order of the pretreatment in the combined studies, especially in biological-chemical/physicochemical methodologies by ascertaining the mechanism of action of each pretreatment method. Nonetheless, it is necessary to determine the environmental impact, cost efficiency, and energy balance of these combined processes to scale the process.



## III.2 Combined Biological and Chemical/Physicochemical Pretreatment Methods of Lignocellulosic Biomass for Bioethanol and Biomethane Energy Production – A Review

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applied microbiology



Review

### Combined Biological and Chemical/Physicochemical Pretreatment Methods of Lignocellulosic Biomass for Bioethanol and Biomethane Energy Production—A Review

Shruthi Meenakshisundaram , Antoine Fayeulle , Estelle Léonard , Claire Ceballos, Xiaojun Liu and André Pauss \*



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Université de Technologie de Compiègne, ESCOM, TIMR (Integrated Transformations of Renewable Matter), Centre de Recherche Royallieu, CS 60319, CEDEX, F-60203 Compiègne, France

\* Correspondence: [andre.pauss@utc.fr](mailto:andre.pauss@utc.fr)

**Abstract:** Lignocellulosic biomass is a low-cost and environmentally-friendly resource that can be used to produce biofuels such as bioethanol and biogas, which are the leading candidates for the partial substitution of fossil fuels. However, the main challenge of using lignocellulosic materials for biofuel production is the low accessibility to cellulose for hydrolysis of enzymes and microorganisms, which can be overcome by pretreatment. Biological and chemical pretreatments have their own disadvantages, which could be reduced by combining the two methods. In this article, we review biological–chemical combined pretreatment strategies for biogas and bioethanol production. The synergy of fungal/enzyme–NaOH pretreatment is the only biological–chemical combination studied for biogas production and has proven to be effective. The use of enzyme, which is relatively expensive, has the advantage of hydrolysis efficiency compared to fungi. Nonetheless, there is vast scope for research and development of other chemical–biological combinations for biogas production. With respect to ethanol production, fungal–organosolv combination is widely studied and can achieve a maximum of 82% theoretical yield. Order of pretreatment is also important, as fungi may reduce the accessibility of cellulose made available by prior chemical strategies and suppress lignin degradation. The biofuel yield of similarly pretreated biomass can vary depending on the downstream process. Therefore, new strategies, such as bioaugmentation and genetically engineered strains, could help to further intensify biofuel yields.

**Keywords:** lignocellulosic biomass; combined pretreatment; biogas; bioethanol; microorganisms

### III.2.1 Introduction

With the current rate of global warming and low supplies of crude oil worldwide, replacement of conventional fuels by biofuels is the only viable alternative for the future

(Antonopoulou et al., 2019). According to World Bioenergy Association, liquid biofuels production has been growing at a rate of 12% and biogas production at a rate of 9% annually in the last two decades. In 2019, 159 billion litres of liquid biofuels and 62.3 billion m<sup>3</sup> of biogas were produced globally. The average energy content of bioethanol is 21.1 MJ/ l and that of biogas is 23 MJ/ m<sup>3</sup> (World Bioenergy Association, 2021). Currently, bioethanol is the most widely used liquid biofuel for transportation as it helps to ensure complete combustion and reduce carbon monoxide emissions (United States Environmental Protection Agency, 2022). Biogas when upgraded into biomethane has several uses, namely heat production, electricity production or co-generation of heat and electricity, and transport fuel production. Since biomethane is energy source identical to natural gas, many countries like France, Germany, Sweden, Switzerland and the Netherlands are injecting it directly into the existing natural gas network (IFP Energies Nouvelles). First generation biofuels from agricultural crops have been disputed due to the food versus fuel debate. Therefore, research activities have been focusing on second generation biofuel from agricultural residues and other cellulosic waste products (Hirani et al., 2018).

Conversion of lignocellulosic biomass (LCB) to biofuels produces net-zero greenhouse gas emission and hence, the biofuels obtained are carbon neutral. Although agricultural biomass is theoretically the most prominently available feedstock for renewable energy, biofuel production at the commercial level is still not very successful (Beig et al., 2021). This is due to the recalcitrance of the complex lignocellulosic biomass structure which is majorly caused by lignin. Lignin needs to be disrupted using a pretreatment step in order to increase the accessibility of microbes/ enzymes to cellulose and hemicellulose for further conversion of fermentable sugars to biofuels. The pretreatment strategies, classified as physical, chemical, biological, and physio-chemical pretreatments employ different mechanisms of action and provide various degrees of polymerization. In the recent decade, there is more interest in using a combination of pretreatments to improve the yield of biofuels obtained while reducing the disadvantages of individual ones. Due to the environment-friendly and low-cost nature, biological pretreatment is a preferred method. Combination with chemicals can help to reduce the residence time and improve the hydrolysis rate. It also mutually reduces the chemical and energy demand. The biological–chemical combination is interesting also because it can reduce the amount of inhibitors generated and therefore, much easier to integrate into the downstream process. Once the biomass is pretreated, it can be subjected to either microbial saccharification

and fermentation to produce ethanol or to anaerobic digestion (AD) to produce biogas (Meenakshisundaram et al., 2021; Shirkavand et al., 2016).

### **III.2.1.1 Anaerobic digestion of lignocellulosic biomass**

In an anaerobic digestion process, complex organic material gets broken down using microorganisms and metabolic pathways under anaerobic conditions. The major products of AD are biogas (made up of 50-65% methane and 35-50% carbon dioxide) and nutrient-rich digestate which can be utilized as fertilizer or soil improver (Abraham et al., 2020; Atelge et al., 2020). The biogas can be upgraded using various technologies to bio-methane (consisting of 95-99% methane) that can be used as a transport fuel, for electricity generation, and as feedstock for chemical industries (Ullah Khan et al., 2017). The global bio-methane market was 1.68 billion USD in 2018 and is expected to reach 2.61 billion USD by 2025 (Zion Market Research, 2019).

The energy efficiency ratio of AD, calculated as energy gain to energy input, exceeds other technologies for energy production from biomass (Paudel et al., 2017). Biogas production is also advantageous since a wide variety of biomass could be utilized and is an economical alternative to tackle some of the environmental concerns (Tabatabaei et al., 2020). Cesaro and Belgiorno (2015) had reviewed the composition and methane potential of different crops and it was observed that maize, sorghum, rice straw, sunflower stalk and wheat straw had the highest methane potential of the reviewed crops (Cesaro and Belgiorno, 2015). Nonetheless, biogas production from lignocellulosic biomass has not been scaled up efficiently despite its high methane potential. This is because AD of lignocellulosic biomass has to overcome various challenges such as resistance of biomass to microbial and enzymatic degradation, and adaptation of existing AD digesters to the high dry solid contents of the particular crops (Sawatdeenarunat et al., 2015).

A problem with using biomass such as straw is that it is a light and dry material, which would increase the solid content in the digester and float. For these light biomass, special mixers would be needed to handle this in AD (Sherrard, 2016). Ma et al. (2019) studied the AD characteristics of individual purified components of lignocellulosic biomass, namely, lignin, cellulose, and hemicellulose. It was reported that lignin and highly crystalline cellulose had a negative correlation with biogas production potential. Whereas co-fermentation of hemicellulose and cellulose reduced the rancidity caused by the fast hydrolysis rate of



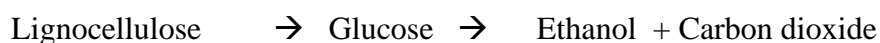
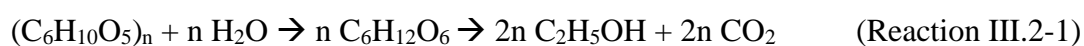
hemicellulose alone and had a positive correlation for biogas production (Ma et al., 2019). Therefore, the ultimate aims of pretreatment processes for improved biogas yield should be to enhance the degradation of lignin content, increase the accessible surface area, and decrease the crystallinity of cellulose while avoiding the degradation or loss of carbohydrates. Consequently, the employment of appropriate pretreatment is the best option for improving the digestion rate and methane production from biomass with varying properties and different digestibilities (Paudel et al., 2017). AD microbes are highly tolerant to inhibitory compounds that may be generated in the process of pretreatment and therefore, detoxification is not needed which makes AD an easier process to utilize lignocellulosic biomass (Zheng et al., 2014).

Apart from the structural recalcitrance, the carbon/ nitrogen (C/N) ratio of lignocellulosic biomass is higher than the optimum C/N ratio (20-30) which limits the efficiency of methane production in AD (Hagos et al., 2017). Different techniques are used to counteract this problem such as pretreatment (Song et al., 2014), co-digestion of nitrogen-rich animal manure with carbon-rich biomass (Comino et al., 2010), high-solid AD (Zhang et al., 2016), bioaugmentation with microorganisms (Y. Li et al., 2018), and nutrient supplementation (Zieliński et al., 2019). Song et al. (2014) observed that total carbon (TC) content decreased when corn straw was chemically pretreated. Although it was higher than the optimum C/N ratio, this helped to decrease the high C/N ratio of untreated lignocellulosic biomass. The methane yield of the chemically pretreated biomass thereby significantly increased compared to untreated samples (Song et al., 2014). Therefore, a successful pretreatment method should improve the digestibility of the biomass for the AD microbes, minimize the formation of inhibitors and be environmentally friendly for avoiding the need for waste disposal. To summarize, more research is required to explore other pretreatment methods as it is the key cost element in the anaerobic digestion of lignocellulosic biomass (Amin et al., 2017).

### **III.2.1.2 Bioethanol production from lignocellulosic biomass**

Bioethanol is the most widely used biofuel to substitute crude oil. Ethanol is produced from lignocellulosic biomass using the three major steps, namely, pretreatment, hydrolysis, and fermentation. Pretreatment increases the porosity of the fiber matrixes, liberates the cellulose from the lignin and hemicellulose complex, and improves the accessibility of enzymes. The pretreated biomass is then hydrolyzed either directly by microbes or with enzymes. Enzymatic hydrolysis offers the advantages of a shorter time period, better yields, and a lower risk of

contamination. Commercial cellulase (extracted from microorganisms) is the most commonly used enzyme for hydrolysis. The hydrolysis process converts polysaccharides into monomer sugars, such as glucose and xylose. Subsequently, sugars are fermented to ethanol (as expressed in reaction III.2-1 below) by the use of different microorganisms. Due to the thermotolerant and wide pH tolerant nature of *Saccharomyces cerevisiae*, it is the preferred organism for ethanol fermentation. Hydrolysis and fermentation can be conducted separately or simultaneously, via the processes called Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) respectively. During the SHF process, each step occurs sequentially at its optimized conditions and the generated sugars are ready when the fermentation step is carried out. SSF process occurs in the same reaction vessel and both reactions occur at the same time. The advantages of SSF include minimized risk of contamination, a shorter time, and less material requirement while the inherent disadvantages are the generation of intermediate products that can inhibit the fermenting microorganisms (Tesfaw and Assefa, 2014; Tuong An Tran et al., 2020).



Acid pretreatment is considered an efficient pretreatment step but it generates various intermediaries like acetic acid, furfural, and 5 hydroxymethylfurfural which inhibit the microorganisms during the fermentation step. Therefore, to reduce the intensity of acid required, a combination with biological pretreatment can be more effective. Zhang et al. (2018) observed an 8.6% increase in ethanol yield from combined pretreatment using white-rot fungi and dilute acid on water hyacinth as compared to sole dilute acid treatment. The higher yield in combined pretreatment was obtained even without the addition of a hydrolysis agent and therefore biological-chemical pretreatment could also help to reduce the cost of ethanol production (Zhang et al., 2018).

The lack of systematic reviews of microbial and chemical/physicochemical combined pretreatments and the knowledge of the advantages of these methods as established by the authors in their previous review (Meenakshisundaram et al., 2021) makes it interesting to review the research on the same for biofuel production. Therefore, this work aims to evaluate combined microbial-chemical/physicochemical pretreatment strategies used for different LCBs based on biogas yield (Subchapter III.2.2) and bioethanol yield (Subchapter III.2.3).

### III.2.2 Comparison of combined pretreatment based on biogas yields

Though there are many reviews from the last two decades studying the various pretreatment strategies of lignocellulosic biomass for biogas production in detail, very few systematically compare the combined pretreatment processes. Biological-chemical combined pretreatment especially for biogas production is not discussed to the best of the authors' knowledge as research is not widely done. Only three research studies (as shown in Table III-3) have utilized a biological-alkaline combined pretreatment strategy so far for enhancing AD of lignocellulosic biomass since both methods help in the breakdown of lignin.

Li et al. (2018) studied the impact of individual components of lignocellulosic biomass on cumulative methane production. Apart from providing a physical barrier to hydrolytic enzymes, the lignin predominantly degrades only in an aerobic environment while being able to persist in an anaerobic environment. Consequently, the lignin offers the maximum resistance in the biomass and is negatively correlated to the biomethane potential (BMP) (W. Li et al., 2018; Ma et al., 2019; Paul and Dutta, 2018). Generally, holocellulose is the portion converted to methane by the mixed anaerobic culture, and therefore, larger degradation of it results in increased carbon dioxide. Therefore, pretreatment of recalcitrant lignocellulosic biomass without losing a major portion of holocellulose is essential to achieve high biogas yield (Alexandropoulou et al., 2017). Without prior treatment, hydrolysis will be slow and a longer retention time will be required to produce a sufficient amount of biogas (Yu and Schanbacher, 2010). The hydrolysis process has a direct interdependence on pH. Acidogenesis and methanogenesis are efficient only in the optimal pH range of 5.5-6.5 and 6.5-8.2 respectively (Mao et al., 2015). Alkaline pretreatment helps to solubilize lignin and neutralize the acidic products released from lignocellulosic biomass. Also, since the residual bases remaining in the pretreated biomass may help to prevent a reduction in pH during the acidogenesis phase and help to increase the efficiency of methanogenesis, it is considered to be more compatible with the anaerobic digestion process (Taherdanak and Zilouei, 2014). The pH value of the AD substrates influences the growth of methanogenic microorganisms and the dissociation of some compounds relevant for the AD process, i.e. ammonia, hydrogen sulfide, and organic acids (Al Seadi et al., 2008). From previous studies, it can be concluded that NaOH is the most widely used alkali pretreatment for lignocellulosic biomass to improve biogas yield (He et al., 2008; Monlau et al., 2012; Yang et al., 2009). Nevertheless, the disadvantage of this method is the possibility of production of  $\text{Na}^+$  ion that can cause inhibition of methanogenesis and lead to

negative environmental impacts on disposal of the effluent (Zheng et al., 2014). Fungal pretreatment has been known as a more environmentally friendly method to improve methane yield of lignocellulosic biomass (Amirta et al., 2006; Lalak et al., 2016; Mackulak et al., 2012). Research on the effect of fungal pretreatment on methane yield has indicated that the results strongly depend on the feedstock (its lignocellulosic content) and the fungi used, as well as on some key operational parameters such as the incubation time, the moisture content, etc. (Alexandropoulou et al., 2017). Therefore, three groups of researchers (as shown in Table III-3) have studied the combination of fungal and alkali pretreatment for improving the methane yield of lignocellulosic biomass.

The results in Table III-3 are represented as [L CH<sub>4</sub>/ kg TS] or [L CH<sub>4</sub>/ kg VS], where TS and VS are total solids and volatile solids content in kilogram (kg) and L CH<sub>4</sub> represents a litre of methane. The percentage of increase in methane yield in combined pretreatment as compared to the single pretreatment step is calculated as given in Eq. III.2-1

$$\% \text{ increase} = \frac{\text{BMP of combined pretreatment} - \text{BMP of sole pretreatment}}{\text{BMP of sole pretreatment}} \times 100 \quad \text{Eq. III.2-1}$$

**Table III-3:** Comparison of combined fungal/ enzyme - alkaline pretreatment based on biomethane yields

Reference	Substrate	Step 1	Step 2	% increase as compared to sole biological pretreatment	Methane yield
Ali and Sun (2015)	Park waste (dry and fresh leaves) + Cattle manure	2.5 % NaOH and 2.5% NH <sub>4</sub> OH (15 d)	<i>Aspergillus terreus</i> and <i>Trichoderma viride</i> (25°C, 7 d)	30 (as compared to untreated biomass)	79.8 L/kgVS (125.9 L/kgVS biogas production)
Alexandropoulou et al. (2017)	Willow sawdust	<i>Leiotrametes menziesii</i> (27°C, 30 d)	1% (w/v) NaOH (80°C, 24 h)	48.9	142.2 ± 0.3 L/kg TS (L. menziesii)
		<i>Abortiporus biennis</i> (27°C, 30 d)		50.1	205.3 ± 0.3 L /kg TS (A. biennis)
Zhao et al. (2018)	Maize Straw	1% (w/v) NaOH (room temperature, 48 h)	<i>Aspergillus sp.</i> (30°C, 10 d)	13.34*	276.29 L/kg TS
			<i>T. harzianum</i> (30°C, 10 d)	22.88*	261.63 L/kg TS
			<i>T. harzianum</i> & <i>Aspergillus sp.</i> (30°C, 10 d)	31.77*	277.99 L/kg TS

			Enzyme from <i>Aspergillus sp.</i> (KY644131) (50°C, 10 d)	25.02*	300.85 L/kg TS
			Enzyme from <i>T. harzianum</i> (KY644130) (50°C, 10 d)	4.14*	285.09 L/kg TS
			Enzyme from <i>T. harzianum</i> & <i>Aspergillus sp.</i> (50°C, 10 d)	-6.71* (decrease)	258.45 L/kg TS

\*calculated by authors according to Eq. III.2-1.

Ali and Sun (2015) observed a 30% increase in methane yield and an 11.9% reduction in CO<sub>2</sub> after combining pretreatment and co-digestion of park waste and cattle manure. By using the fungal pretreatment (*L. Menziesii* and *A. biennis*) as the first step in the combined pretreatment, Alexandropoulou et al. (2017) were able to use a lower concentration of NaOH for the treatment in the subsequent step as compared to Ali and Sun (2015). Though *L. menziesii* had higher lignin degradation than *A. biennis*, it produced lesser BMP than *A. biennis* as the portion of the holocellulose (higher cellulose degradation observed) which could be converted to methane by the mixed anaerobic culture was degraded, resulting in higher CO<sub>2</sub> content. This iterates the fact that low cellulose uptake efficiency is equally important as lignin degradation (Alexandropoulou et al., 2017). Zhao et al. (2018) compared fungi (*T. harzianum* and *Aspergillus sp.*) and their secreted enzymes as a biological pretreatment step after NaOH treatment of maize straw. In this combined pretreatment study, the enzymes compared better than their fungi counterparts in producing methane yield. Zhao et al. (2018) remarked that in general when fungi are used, it resulted in nutrients from the lignocellulosic substrate being consumed for their growth and thus reducing the methane yield. Whereas, when enzymes derived from the fungi are used, they help to convert cellulose and hemicelluloses into reducing sugars and other small molecules that are readily used by microorganisms in the AD process and thereby increasing the methane yield. An exception was noted when a combination of *T. harzianum* and *Aspergillus sp.* and when a combination of their secreted enzymes was used along with NaOH pretreatment. The two fungi inhibited each other and slowed the decomposition of the substrate while the combination of their enzymes showed a contrast effect, resulting in higher hemicelluloses removal. This resulted in an irregularity where the combination of two fungi with NaOH produced better methane yield than their secreted enzymes (Zhao et al., 2018). However, it has been observed that even if there is a possibility of some drawbacks in the combined pretreatment, it is an efficient process to improve biogas and biomethane content.

The hydrolysis of lignocellulosic biomass is dependent on the lignin to cellulose ratio. The low lignin to cellulose (L/C) ratio implies a higher degree of digestibility (Scharer and Moo-Young, 1979). An improvement was noted in the L/C ratio from 0.13 for untreated maize straw to 0.10 for 1% NaOH treated maize straw (Zhao et al., 2018). From the values given in the study by Alexandropoulou et al. (2017), the L/C ratio for willow sawdust is calculated to be 0.81. When treated with the *Leiotrametes menziesii* and NaOH, the ratio was lowered to 0.67

while treatment with *Abortiporus biennis* and NaOH further lowered the ratio to 0.52. The methane yield values obtained for the same (as seen in Table III-3) corroborate the statement that with lower L/C value, higher the degree of digestibility and thereby higher methane yield is obtained. On that account, such synergetic pretreatment methods have to further be formulated and developed to enhance biogas yields that are cost-effective and sustainable compared to conventional ones.

### III.2.3 Comparison of combined pretreatment based on bioethanol yields

*Saccharomyces cerevisiae* is the most employed strain used for fermentation of enzymatic hydrolysate to ethanol. This is because *S. cerevisiae* tolerates higher temperatures and a wide range of pH (with the acidic pH being the optimum), which makes its fermentation less susceptible to contamination than bacteria. It is also known to tolerate ethanol better than other ethanol-producing microorganisms. *S. cerevisiae* shows a broad substrate utilization which is important for commercially viable ethanol production. The media used for the fermentation, in general, consists of yeast extract, peptone, NH<sub>4</sub>Cl, KH<sub>2</sub>PO<sub>4</sub>, and MgSO<sub>4</sub> as supplementation of exogenous nitrogen sources enhances sugar utilization and ethanol production. *S. cerevisiae* is also GRAS (generally regarded as safe) for human consumption and therefore, easier to handle (Tesfaw and Assefa, 2014).

Separate hydrolysis and fermentation (SHF) were used conventionally as optimal temperatures for enzymatic hydrolysis and fermentation are different. While hydrolysis takes place efficiently at 45-50°C, the best conditions for fermentation are at 28-35°C. However, simultaneous saccharification and fermentation (SSF) has taken precedence in recent times due to its advantages. Indeed, SSF is considered a less capital-intensive process due to its lower overall processing times, eliminating the need for separate reactors and providing higher ethanol yields. The higher ethanol yields correspond to more conversion of xylose to xylitol under SSF conditions (Monrroy et al., 2010).

From Table III-4, it can be seen that the ethanol yields are either detected using HPLC or GC-FID techniques. The percentage of the theoretical yield is calculated by dividing the ethanol amount obtained (g) by the amount of hexoses in pulp (g), assuming that all the hexoses were available for fermentation, with a fermentation stoichiometric yield of 0.51 g (ethanol)/ g (hexose) and multiplied by 100% (Muñoz et al., 2007). The ethanol yield increase is calculated as shown in equation III.2-2.



$$\text{Ethanol yield increase} = \frac{\text{Ethanol conc. after combined pretreatment} - \text{Ethanol conc. after sole pretreatment}}{\text{Ethanol conc. after sole pretreatment}} \times 100. \text{ Eq. III.2-2}$$

### III.2.3.1 Combined biological-alkaline pretreatment

Fissore et al. (2010) and Salvachúa et al. (2011) studied biological followed by alkaline treatment of wood and wheat straw respectively. Brown rot pretreatment of *Pinus radiata* wood chips leads to carbohydrate degradation while lignin is not severely attacked. The alkaline pretreatment of bio treated wood chips further favored the removal of short cellulose chain, leaving a high amount of residual lignin in the pulp. The low pulp yield and low carbohydrate retention led to a decrease in enzymatic hydrolysis and fermentation efficiency of the wood in biochemical treatment as compared to sole alkaline pretreatment (Fissore et al., 2010). *I. lacteus* and *P. subvermispora* pretreatment of wheat straw showed intermediate levels of glucose consumption, and no inhibitors of the fermenting yeast were generated in the process of alkaline pretreatment. Consequently, there was a 90% conversion of glucose to ethanol, resulting in an ethanol yield of 62% in both cases (Salvachúa et al., 2011).

### III.2.3.2 Combined biological-acid pretreatment

Zhang et al. (2018) compared the combination of biological and mild acid pretreatment with the combination of biological and dilute alkaline pretreatment for water hyacinth biomass. It was observed that even though alkaline treatment showed the most effective lignin removal ability, combined biological - acid treatment resulted in higher reducing sugar content as more cellulose was preserved (K. Zhang et al., 2018). Ma et al. (2010) also confirmed that combined biological-acid pretreatment stimulated better ethanol fermentation by increasing glucose concentration, decreasing the fermentation inhibitors, or producing fermentation accelerants. Therefore, microbial-acid pretreatment is considered to be better for ethanol production from water hyacinth. Ma et al. (2010) studied SHF while Zhang et al. (2018) studied SSF with *Saccharomyces cerevisiae* on water hyacinth pretreated with combined biological-acid treatment. Due to the addition of cellulase enzyme and conducting hydrolysis and fermentation subsequently (SHF) at optimized conditions, Ma et al. (2010) obtained a greater increase in ethanol yield as compared to Zhang et al. (2018).

**Table III-4:** Ethanol yield from different biological-chemical/physicochemical pretreatment strategies

Substrate	1 <sup>st</sup> step	2 <sup>nd</sup> step	Hydrolysis and Fermentation [Ethanol concentration detection technique]	Ethanol yield increase (%)	Ethanol concentration (% theoretical ethanol yield)	Reference
<b>Biological – Alkaline Pretreatment</b>						
<i>Pinus Radiata</i>	<i>Gloeophyllum trabeum</i> (27°C, 28 d)	25% w/ w NaOH (180°C, 5 h)	SHF: 5% substrate consistency. Celluclast (20 FPU/g) and $\beta$ -glucosidase (40 UI/g) (50°C, 24 h, 150 rpm). 3 g/L <i>Saccharomyces cerevisiae</i> IR2-9a (40°C, 96 h, 150 rpm). [GC-FID]	-37.5%* compared to alkaline alone	99.55 mL/kg wood (33.98%)	Fissore et al. (2010)
Wheat straw ( <i>Triticum aestivum</i> )	<i>P. subvermispora</i> (28°C, 21 d)	0.1% NaOH (5% w/v) (50°C, 1 h, 165 rpm)	SHF: Cellulase (15 FPU/g) and xylanase (30 U/g) (50°C, 60 h, 165 rpm). 0.5 g/L <i>Saccharomyces cerevisiae</i> (Fermentis LPA 3035) (32°C, 24 h, 200 rpm). [GC-FID]	79.41%* compared to alkaline alone	122 $\pm$ 8 mg/g substrate (62%)	Salvachúa et al. (2011)
	<i>I. lacteus</i> (28°C, 21 d)			80.88%* compared to alkaline alone	123 $\pm$ 5 mg/g substrate (62%)	

Biological – Acid Pretreatment						
Water Hyacinth ( <i>Eichhornia crassipes</i> )	<i>Echinodontium Taxodii</i> (28°C, 10 d)	0.25% H <sub>2</sub> SO <sub>4</sub> (100°C, 1 h)	SHF: 2% substrate consistency. Cellulase (30 FPU/g) (50°C, 48 h). 0.3% v/v activated <i>Saccharomyces cerevisiae</i> (40°C, 72 h, 100 rpm for first 8 h). [HPLC]	31.51%* (1.34-fold higher) than sole acid	0.192 g/g of dry material (sole acid = 0.146 g/g of dry material)	Ma et al. (2010)
<i>Glycyrrhiza uralensis</i> Fisch. Ex DC (GUR)	<i>Phanerochaete chrysosporium</i> (28°C, 21 d)	2.5% H <sub>2</sub> SO <sub>4</sub> (100°C, 2.5 h)	Cellulase (30 FPU/g) (50°C, 48 h) Heterotrophic cultivation of <i>C. protothecoides</i> for microalgal oil production. (28°C, 7 d, 200 rpm) [GC-MS]	1.34-fold higher than acid only treated sample	1.66 g/L (oil content)	Gui et al. (2013)
<i>Glycyrrhiza uralensis</i> Fisch. Ex DC (GUR)	<i>Phanerochaete chrysosporium</i> (28°C, 21 d)	2M acetic acid (100°C, 3 h)	Cellulase (40 FPU/g) (50°C, 48 h). Heterotrophic cultivation of <i>C. protothecoides</i> (28°C, 120 h, 200 rpm) [GC-MS]	1.54*-fold higher than acid only treated sample	1.91 g/L (oil content)	Gui et al. (2014)
Oil palm empty fruit bunches (OPEFB)	<i>Pleurotus floridanus</i> LIPIMC996 (31°C, 28 d)	Ball milled at 29.6/s for 4 mins. Phosphoric acid treatment (50°C, 5 h)	SSF: Enzymatic hydrolysis (60 FPU/ g cellulose). <i>Saccharomyces cerevisiae</i> CBS 8066 (35°C, 48 h, 130 rpm). [HPLC]	7.39%* increase than sole acid treatment	21.8 g/L (62.8%)	Ishola et al. (2014)

Water hyacinth	<i>Phanerochaete chrysosporium</i> (30°C, 60 h, 150 rpm)	1% H <sub>2</sub> SO <sub>4</sub> (100°C, 1 h)	6 g/L <i>Saccharomyces cerevisiae</i> (30°C, 24 h, 120 rpm) [GC]	8.61% increase than sole acid pretreatment	1.40 g/L	Zhang et al. (2018)
<b>Biological – Organosolv Pretreatment</b>						
Sapwood of beech ( <i>Fagus crenata</i> )	<i>C. subvermispora</i> FP90031 (28°C, 56 d)	60% (v/v) ethanol solution (180°C, 2 h)	SSF: Cellulase (10 FPU/ 0.25 g). 10% v/v <i>S. cerevisiae</i> AM12 (40°C, 96 h, 100 rpm). [GC-FID]	1.6 times higher than that of ethanolysis alone	0.176 g/g of wood	Itoh et al. (2003)
<i>Pinus radiata</i> woods chips	<i>Ceriporiopsis subvermispora</i> (27°C, 30 d)	60% ethanol in water solvent (200 °C, 1 h) (H-factor: 11,360); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75 °C, 1 h)	SHF: Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50°C, 72 h, 150 rpm). <i>Saccharomyces cerevisiae</i> Y-1528 (30°C, 48h, 150 rpm). [GC-FID] SSF: 2% substrate consistency. Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50°C, 72 h, 150 rpm). <i>Saccharomyces cerevisiae</i> Y-1528 (37°C, 48h, 150 rpm). [GC-FID]	SHF: 61.90%* SSF: 458%* compared to control	SHF: 136 g/kg wood (37%) SSF: 162 g/kg wood (44%)	Muñoz et al. (2007)

<i>Acacia dealbata</i> woods chips	<i>Ganoderma australe</i> (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 10,920); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)	SHF: Cellulase (20 FPU/g glucan) and $\beta$ -glucosidase (40 CBU/g glucan) (50°C, 72 h, 150 rpm). <i>Saccharomyces cerevisiae</i> Y-1528 (30°C, 48h, 150 rpm). [GC-FID] SSF: 2% substrate consistency. Cellulase (20 FPU/g glucan) and $\beta$ -glucosidase (40 CBU/g glucan). <i>Saccharomyces cerevisiae</i> Y-1528 (37°C, 48h, 150 rpm). [GC-FID]	SHF: -7.14%* SSF: 4.28%* compared to control	SHF: 143 g/kg wood (48%) SSF: 195 g/kg wood (65%)	Muñoz et al. (2007)
<i>Pinus radiata</i>	<i>Gloeophyllum trabeum</i> (27°C, 28 d)	60% ethanol in water solvent (200°C, 1 h)	SSF: Celluclast (20 FPU/g) and $\beta$ -glucosidase (40 UI/g) (50°C, 24 h, 150 rpm). 3 g/L <i>Saccharomyces cerevisiae</i> IR2-9a (40°C, 96 h, 150 rpm). [GC-FID]	44.6%	210 mL/kg wood (72%)	Fissore et al. (2010)
<i>Pinus radiata</i> wood chips	<i>Gloeophyllum trabeum</i> ATCC 11539 (25°C, 21 d)	Biopulp: 95% ethanol in water solvent (60:40 v/v ratio) with 0.13% H <sub>2</sub> SO <sub>4</sub> (w/v) (185°C, 18 min) Control pulp: 95% ethanol in water	SSF: 10% substrate consistency. Celluclast (20 FPU/ g), $\beta$ -glucosidase Novozymes 188 (40 IU/ g). 6.0 g/L <i>Saccharomyces cerevisiae</i> IR2T9 (40°C, 96 h, 150 rpm). [GC-FID]	Similar yield in both control pulp and biopulp	161 g/kg wood (63.8%)	Monroy et al. (2010)

		solvent (60:40 v/v ratio) with 0.13% H <sub>2</sub> SO <sub>4</sub> (w/v) (200°C, 32 min)				
Japanese cedar ( <i>Cryptomeria japonica</i> )	<i>Phellinus sp.</i> SKM2102 (28°C, 56 d)	Ethanol/ lactic acid/ water (40:10:50, w/w) (190°C, 30 mins)	SSF: Meicelase (10 FPU/ 0.25 g). 10% v/v <i>S. cerevisiae</i> AM12 (35°C, 72 h, 100 rpm). [GC-FID]	NA	8.94 g/L (28.3%)	Baba et al. (2011)
	<i>C. subvermispora FP-90031-sp</i> (28°C, 56 d)	Ethanol/ lactic acid/ water (40:10:50, w/w) (200 °C, 1 h)			9.82 g/L (31.1%)	
<b>Biological – Steam Explosion Pretreatment</b>						
Sawtooth oak, corn, and bran	<i>Lentinula edodes</i> (120 d)	Steam explosion (214°C, 5 mins, 20 atm)	SSF: 0.1 g enzyme/ g substrate Meicelase (45°C, 48 h, 140 strokes/min). <i>Saccharomyces cerevisiae</i> AM 12 (40°C, 24 h, 100 rpm). [HPLC]	49.68%* increase compared to spent shitake mushroom medium	23.8 g/L (87.6%)	Asada et al. (2011)

\*As calculated by authors using the data given in the research article.

\*\* [GC-FID: Gas chromatography - detector; HPLC: High performance liquid chromatography].

Pretreatment of oil palm empty fruit bunches (OPEFB) using fungal pretreatment, phosphoric acid pretreatment and the combination of both methods produced the highest ethanol concentrations of 6.8 g/L, 20.3 g/L, 21.8 g/L respectively. However, the percentage of the theoretical yield of ethanol was 27.9% in 72 hours in the case of fungal pretreatment, and 89.4% and 62.8% in 48 hours of phosphoric acid and combined pretreatment respectively. This is because, in the case of combined pretreatment, there was a material loss of 63.6% which could decrease the ethanol yield. Still, a greater approach towards theoretical yield is observed in phosphoric acid alone or combined pretreatment than in sole fungal pretreatment (Ishola et al., 2014). Gui et al. (2013 and 2014) worked on combined *Phanerochaete chrysosporium* - acid pretreatment (2.5% H<sub>2</sub>SO<sub>4</sub> and 2M acetic acid respectively) on *Glycyrrhiza uralensis* Fisch. Ex DC (Chinese licorice). Biomass growth and oil production by *C. protothecoides* were seen to be better in the co-treated samples than in sole acid pretreatment. Combined biological and acetic acid seemed slightly better than combined biological and sulfuric acid pretreatment. This could be due to either reduction in the number of inhibitors generated or due to an increase in production of certain growth accelerants (such as proteins, amino acids, or other components) during combined pretreatment (Gui et al., 2014, 2013).

### **III.2.3.3 Combined biological – organosolv pretreatment**

Fungal-organosolv is the most studied biological-chemical pretreatment for ethanol production. Itoh et al. (2003) compared 4 strains for fungal pretreatment of sapwood of beach and their ethanol yield. It was observed that fungal treatments with *D. squalens* and *C. subvermispora* improved the ethanol yield while *P. ostreatus* and *C. versicolor* did not show a significant increase. The yield of ethanol increased with an increase in ethanolysis temperature but combining with fungal treatment helped to decrease the ethanolysis temperature to below 200°C, thereby saving 15% of the electricity needed for ethanolysis. The fungal pretreatment with *C. subvermispora* improved the separation of cellulose and hemicellulose components on ethanolysis at 180°C leading to 82% recovery of carbohydrates and thereby significant increase in ethanol yield (Itoh et al., 2003).

Muñoz et al. (2007) studied bio-organosolv pretreatment on both *P. radiata* and *A. dealbata* wood chips. Due to the low lignin content in *A. dealbata*, the glucan to glucose conversion was rapid and therefore, fermentation led to higher ethanol yield than *P. radiata*. Overall, there was still low wood to ethanol conversion because of low pulp yield in the case

of *A. dealbata* and due to high residual lignin content in *P. radiata* pulps. The low ethanol yield was not associated with inhibitors' action but with low pulp consistency during enzymatic hydrolysis. This could be concluded because most of the yeast inhibitors generated from the pretreatment step are removed during the washing step. During SHF and SSF of both *P. radiata* and *A. dealbata*, the effect of ethanol catabolism was observed. This is attributed to low pulp consistency (2%) which leads to low concentrations of fermentable carbohydrates in the medium to be used by the yeast. Due to the low sugar concentration, substantial catabolic ethanol oxidation by the yeast and thereby low ethanol yields due to 'diauxic shift' are caused. The diauxic shift occurs due to a change in the metabolism of *S. cerevisiae* from fermentation to respiration when glucose is exhausted. Comparatively, SSF produced higher ethanol yields of bio-organosolv pretreated *P. radiata* and *A. dealbata* wood chips after a longer processing time as compared with SHF. This could be a consequence of a lower enzymatic hydrolytic rate under SSF conditions. However, untreated *P. radiata* wood chips produced higher ethanol yield under SHF conditions than at SSF conditions. This was attributed to high lignin content in *P. radiata* which deterred the enzyme action at low temperature (37°C) used in SSF as compared to optimized conditions in SHF (hydrolysis at 50°C and fermentation at 30°C) (Muñoz et al., 2007). Similarly, biotreatment was observed to improve the hydrolysis rate and obtain a higher glucan to glucose conversion in the case of the study done by Fissore et al. (2010). Moreover, the organosolv pulping was better than alkaline pulping of biotreated samples and resulted in 72% of the maximum possible ethanol yield. This is because of the selective delignification action by organosolv which helped to retain more carbohydrates in the pulp, while alkaline delignification favored the removal of short cellulose chains (Fissore et al., 2010). Monrroy et al. (2010) did a very similar study as Fissore et al. (2010) and found out that biotreated and control pulps when subjected to organosolv process produced similar ethanol yields. However, the organosolv process conditions required for biotreated pulp were much less severe because of improvement in solvent accessibility. As is stated in a lot of reviews, physical structural features that are relevant for conversion of LCBs to liquid fuel such as reduction in lignin content, decrease in cellulose crystallinity, increase in pore volume and decrease of particle size are better in biotreated biomass compared to control, which improves solvent accessibility (Monrroy et al., 2010). Similarly, when white-rot fungi were studied in combination with mild ethanolysis without acid, a significant improvement in the ethanol yield of softwood was observed (Baba et al., 2011).



### III.2.3.4 Combined biological-steam explosion pretreatment

Fungi-steam explosion pretreated biomass produced a water extractive fraction that contained 5-hydroxymethylfurfural (HMF) and furfurals apart from sugars and organic acids. Therefore, to achieve efficient ethanol production, water extraction was necessary to remove the HMF and furfurals which are inhibitors to alcohol fermentation. In the study by Asada et al. (2011), without water extraction, there was glucose accumulation observed during SSF but no ethanol was produced. A probable explanation was that *S. cerevisiae* was inhibited by water-soluble lignin, furfural, HMF, and organic acids. Consequently, a water extraction step was included, and to reduce the cost of ethanol separation from the fermentation broth, SSF was conducted with a high substrate concentration. It was also observed that the ethanol conversion rate of 87.6% was achieved at 100 g/L of substrate concentration while a further increase in substrate concentration to 300 g/L produced the highest ethanol yield of 38.8 g/L after 60 h of incubation. On the other hand, it was observed that the *S. cerevisiae* stopped the uptake of glucose at 35 h of incubation, after which with an increase in incubation time, there was just glucose accumulation in the fermentation broth. Also, beyond 100 g/L of substrate concentration, the ethanol conversion rate declined as lignin separated at high concentration inhibited the growth of *S. cerevisiae*. Therefore, a substrate concentration of 200 g/L was estimated to be optimum to produce the highest ethanol concentration (Asada et al., 2011).

From the various articles analyzed in this section, fermentation efficiency and production costs are seen to be dependent on various factors like substrate consistency, total enzyme load to substrate ratio, and glucose loss due to fungal decay. High substrate concentration is observed to inhibit enzyme action due to high residual lignin content and increase of inhibitory substance concentration while producing problems such as mixing and mass transfer in the fermentation process. The hydrolysis rate depends on the total enzyme to substrate ratio, so lowering the substrate consistency while increasing the enzyme load, increases the yield but also the cost of the process. Therefore, fermentation strategies need to be improved to have a high substrate concentration to produce a higher ethanol yield per batch while reducing the cost. Considering the cost of ethanol separation from the fermentation broth, strategies to continuously remove inhibitory materials in the fermentation broth need to be developed (Asada et al., 2011; Fissore et al., 2010).

### III.2.4 Properties of the employed microorganisms in the pretreatment step

From the articles reviewed in Meenakshisundaram et al. (2021), it has been observed that 40 microbial strains have been used for combined biological-chemical/physicochemical pretreatment studies, of which 33 are fungi and 7 bacteria (represented in green in the phylogenetic tree in Figure III-1). Usage of bacteria for the degradation of lignin has not been extensively studied compared to fungal degradation. Bacteria produce secondary metabolites and use extracellular enzymes for the breakdown of lignocelluloses but the delignification was classically reported as slower and more limited (Janusz et al., 2017). Bacteria from phyla Proteobacteria, Actinobacteria, and Firmicutes are known to produce the ligninolytic enzymes and are major decomposers of lignocelluloses in soils. Actinobacteria and Proteobacteria use lignin depolymerization, aromatic compound catabolism, and specific product biosynthesis for lignin breakdown (Chukwuma et al., 2021). It is to be noted that Actinobacteria are not represented in Figure III-1 as they are mostly studied for enzyme production by growing on pretreated biomass than to use them in the pretreatment step for LCB degradation. This is because they are mostly cellulolytic bacteria. Similarly, *Bacillus sp.* belonging to Firmicutes, though known for its lignin-degrading abilities, is being exploited for cellulolytic enzymes increasingly. This is because its mechanism is still unknown and lignin-degrading rate is generally lower than fungi (Dobrzyński et al., 2022; Yang et al., 2013). *Pseudomonas putida* of the phyla beta-Proteobacteria and *Cupriavidus basilensis* B-8 of the gamma-proteobacteria phyla is well known for its degrading abilities by producing manganese peroxidase. Bacteroides such as *Sphingobacterium* produces manganese superoxide dismutase, which uses a hydroxyl radical mechanism to oxidize lignin. These findings on the mechanism of LCB degradation indicate that the use of bacteria for lignocellulosic biomass pretreatment could prove to be more important than previously thought; especially because bacterial growth time is lower than that of fungi. It is interesting to study more bacteria and their enzymes for biofuel production as they can be engineered and due to their ability to exist in a range of environmental conditions (Chukwuma et al., 2021; Janusz et al., 2017; Zhuo et al., 2018).

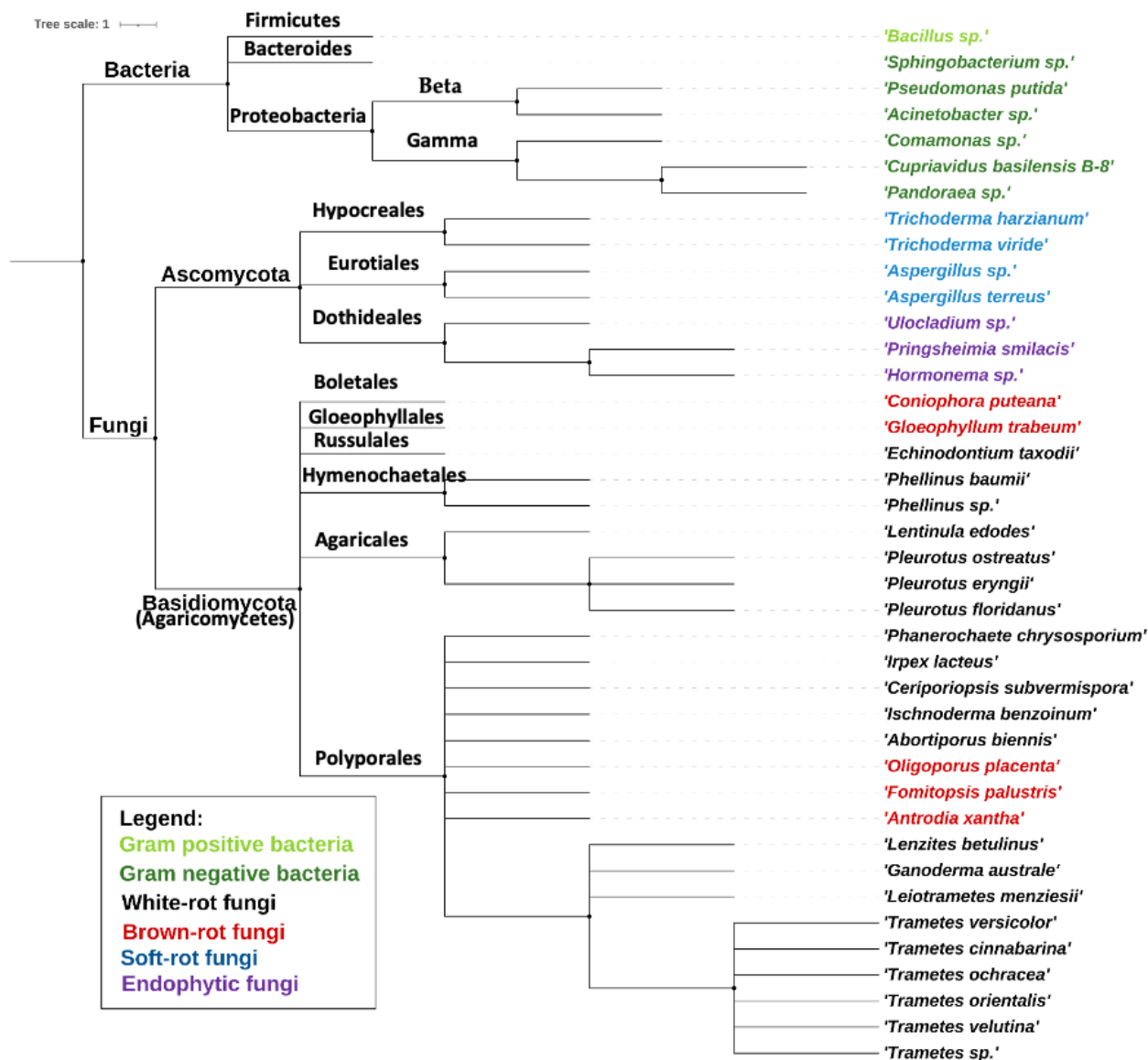


Figure III-1: Phylogenetic tree of the microbes used in combined pretreatment of lignocellulosic biomass (Only the microbes used in the studies compared in the review of Meenakshisundaram et al. (2021) are represented here. The data was input in the NCBI taxonomy browser and a phylip tree file was generated. This was then uploaded on the iTOL website and the phylogenetic tree image was generated.).

The fungi that include species from Ascomycota and Basidiomycota phyla are well known for their decaying capacities using ligninolytic enzymes. Most soft rot fungi (shown in Figure III-1 in blue and purple) belong to Ascomycota and they typically attack the outer surface of the wood in relatively wet environments, leading to softening of wood. The enzymes laccases and peroxidases that are produced by soft rot fungi are unspecified, more limited in

function, and very little is known about their degradation mechanism (Andlar et al., 2018). Endophytic fungi, on the other hand, do not cause any harm to the host plant and need healthy plants to sustain their life while helping the host to resist diseases and droughts. Endophytes are an emerging group of fungi used for biofuel production as they produce hydrocarbons while utilizing plant components. Their contribution to the formation of crude oil is proven. Therefore, it is interesting to study the pathways in which endophytes help in biofuel production and to utilize the secondary products for industrial applications (Strobel, 2018). Basidiomycota phylum is in very high predominance and more particularly the class Agaricomycetes (as can be seen from Figure III-1), which are well known as wood decomposing fungi. Agaricomycetes are mushroom-forming fungi that display two main modes of deadwood decay, i.e., white rot and brown rot. White-rot fungi degrade lignin, and parts of hemicelluloses and cellulose while leaving a bleached residue. Brown rots, on the other hand, attack the hemicellulose and cellulose with only minor modification to lignin, leaving a brownish residue (Krah et al., 2018). Brown rot fungi are the major component of forest soils and litter and to metabolize the holocellulose component of wood, they secrete both enzymatic and non-enzymatic degradative metabolites. The molecular size of enzymes secreted is too large to penetrate the pore structure of the wood. Therefore, it is hypothesized that low molecular weight compounds diffuse into the cell wall and are used to catalyze the production of hydroxyl radicals in a Fenton-like mediated reaction. Most brown-rot fungi produce endoglucanases to cleave the  $\beta$ -1,4 glucosidic linkages, and  $\beta$ -glucosidases to hydrolyze cellobiose or other short oligosaccharides. They also produce several endo-xylanases and  $\beta$ -xylosidases, which are needed for the breakdown of hemicelluloses. Brown rot fungi such as *Coniophora puteana* can produce cellobiohydrolase, an exo-cleaving enzyme that acts on cellulose. Because of their selective holocellulose degrading ability, only five fungi used in the studies reviewed here are brown rot (shown in red in Figure III-1). The white-rot fungi also use both enzymatic and non-enzymatic systems to attack preferentially the hemicellulose and lignin in wood. White rot fungi use enzymes like lignin peroxidase, manganese peroxidase, and laccase for the ligninolytic activity while endoglucanases and exo-glucanases are both produced to synergistically act on crystalline cellulose. The metals and radical ions generated from enzymatic action help in the non-enzymatic penetration mechanism into the wood (Baldrian and Valášková, 2008; Janusz et al., 2017; Krah et al., 2018; Wan and Li, 2012; Wei, 2016). The majority of strains studied for lignocelluloses degradation belong to the order of Polyporales within the class Agaricomycetes. Six strains of

the Polyporales belong to the *Trametes* genus, which belongs to white-rot fungi. The higher number of studies using white-rot fungi is due to the preference for highly selective lignin biodegradation to ensure a cellulose-rich substrate for biofuel production. Considering that the type of lignocellulolytic enzymes produced is limited to the type of strain, one of the best approaches to improve biofuel production of pure culture pretreatment is to combine it with other pretreatment methods (Wei, 2016).

### **III.2.5 New trends in microorganisms used in downstream process**

Existing commercial fungal pretreatment technology for the AD process only uses aerobic fungi, which could pose facility-related costs, mainly for sugar production because of low sugar efficiency and high retention time. On the other hand, using anaerobic fungi could reduce the long retention time because of its ability for simultaneous biological pretreatment and AD process. This would also help lower the capital investments required for a separate aerobic pretreatment reactor. The potential anaerobic fungi for lignocellulosic biomass degradation can be selected based on those found in digestive tracts and feces of ruminant and non-ruminant herbivores (Tabatabaei et al., 2020). For example, Dollhofer et al. (2015) studied anaerobic fungi isolated from rumen fluid of a cow and of a chamois such as *Neocallimastigomycota* which help in the decay of the major portion of consumed fodder. The lignin is mechanically disintegrated by the growth and expansion of the rhizoids of the Neocallimastigales, which makes cellulose and hemicellulose accessible for further attacks. Further, they possess highly efficient cellulases and several enzymes needed to catabolize hemicelluloses. The carbohydrates are further metabolized to produce compounds that provide energy in the form of ATP for fungal growth and which are also possible substrates for methanogens. This syntrophic interaction is more advantageous because the energy in biomass is captured and converted mostly to methane without loss by respiration as during an aerobic pretreatment process. Though these anaerobic fungi have been observed to accelerate the degradation of dry matter and produce an initial increase in biogas production, it is followed by an increase in the concentration of volatile fatty acids (VFAs). This leads to a requirement of separate hydrolytic and fermentation phases in an AD process. Therefore, anaerobic lignocellulolytic fungi could be used as a cost-efficient method to circumnavigate the bottlenecks possessed by hydrolysis (Dollhofer et al., 2015). Cheng et al. (2009), Jin et al. (2011), and Procházka et al. (2012) have all reported an increase in methane production in 3-7 days when a simple co-culture of anaerobic fungus and methanogens are used to treat LCBs.

Another promising strategy that has demonstrated improved methane yield from LCBs is addition of rumen fluid which contain several microbial communities which are able to secrete multiple digestive enzymes at the same time such as cellulase, hemicellulose and  $\beta$ -glycosidase. These enzymes help to gradually degrade the LCB components to VFAs (Ferdeş et al., 2020). These projects have not been translated into large-scale levels due to the difficulty of using strictly anaerobic microbes and keeping the digesters strictly anaerobic throughout the fermentation. Further research is required to study the best conditions for more such co-cultures (Cheng et al., 2009). Conventionally, oxygen is known to inhibit AD process but recently, microaeration which introduces a very limited supply of oxygen is seen to be an alternative technique to improve the AD efficiency. Microaeration enhances the abundance of facultative bacteria such as the phylum Firmicutes during the hydrolysis phase, and this higher growth rate results in higher hydrolytic enzymes. Consequently, a shorter lag phase and better hydrolysis rate are observed. The introduction of limited amounts of oxygen were not lethal to methanogens but led to the increase of oxytolerant genus due to acclimatization. These shifts in the microbial community structure are responsible for improvement of anaerobic digestion efficiency of cellulosic substrates (Fu et al., 2016).

The direct addition of selected strains or mixed cultures to anaerobic digesters is called bioaugmentation, which helps in improving the catabolism of resistant material such as lignocellulosic biomass. It is also an environment-friendly, cost-effective form of biological pretreatment, even though some view it as an improved inoculation method to increase the methane yield (Wei, 2016). Considering bioaugmentation as a pretreatment step, Hu et al. (2016), Mulat et al. (2018), and Sträuber et al. (2015) combined bioaugmentation and chemical pretreatment for enhancing biomethane yields. Though, the maximum methane yield was only slightly improved in these studies, the lag phases were slightly shorter than their non-bioaugmented counterpart (Mulat et al., 2018).

For ethanol fermentation, direct microbial conversion (DMC) and simultaneous saccharification and co-fermentation (SSCF) strategies are being tested out. In DMC, a mono- or co-culture of microorganisms is used for cellulase production, biomass hydrolysis, and ethanol fermentation in a single reactor. This reduces the capital investment required as bacteria such as *Clostridium thermocellum* and some fungi including *Neurospora crassa*, *Fusarium oxysporum*, and *Paecilomyces sp.* have been shown to produce cellulases and help in the fermentation of cellulose directly to ethanol. But due to the long fermentation period required

(3-12 days) and due to low ethanol yields, it is not yet regarded as an efficient process. SSCF is different from DMC as it uses a combination of microorganisms sequentially at different fermentation periods for better utilization of sugars (Sarkar et al., 2012). Though *S. cerevisiae* is known to be a robust organism for ethanol production, it cannot utilize the pentose sugar – xylose. Other yeasts such as species belonging to the *Pichia* and *Candida* genera can utilize C-5 sugars but their ethanol production rate is very low compared to *S. cerevisiae*. So, for LCBs like sugarcane bagasse and rice straw, which contain more than 20% of xylose, *S. cerevisiae* is employed in the first phase of fermentation for utilization of hexose sugars, followed by *Candida shehatae* in the second phase for pentose utilization. Here, the microorganism is chosen based on its compatibility to pH and temperature during the operating phase (Meethit et al., 2016). Still, the ethanol yields achieved are not high and therefore genetic engineering has been applied to develop robust strains capable of fermenting pentoses to obtain higher yields. Several genetically modified microorganisms such as *P. stipitis* BCC15191, *P. stipitis* NRRL Y-7124 recombinant *E. coli* KO11, *C. shehatae* NCL-3501, *S. cerevisiae* ATCC 26603 have been developed for a wide range of monomer utilization (Sarkar et al., 2012). Kun et al. (2019) has also comprehensively reviewed the progress and possibilities in strain engineering of filamentous fungi for improved enzyme production that help in the degradation of lignocellulosic biomass. More research on such genetically modified fungi strains and their application in effective submerged or solid-state fermentation processes can help to establish a bio-based economy on a greater scale (Kun et al., 2019).

### **III.2.6 Challenges and possible solutions**

The major challenges of scaling up lignocellulosic biofuels is the cost factor. Pretreatment accounts for 17% of the production costs. Since the pretreatment steps sometimes involve acids/ alkali or solvents, or operation at high temperature and pressure, special corrosion resistant equipments are needed which add to the capital costs. To reduce the operational costs, solvent losses need to be minimized while maximizing biomass loading. Therefore, optimization of operational parameters is needed to identify process parameters that have the greatest economic impact and address those issues. Since the energy content of LCBs are not fully utilized in only biogas or bioethanol production, combination of anaerobic digestion and fermentation process from the same pretreated biomass could help to overcome these limits (Cheah et al., 2020). Experimental results from Cesaro and Belgiorno (2015) show that the stillage obtained as a by-product of fermentation to produce ethanol still has the organic

potential to be transformed into methane. Moreover, fermentation acts as pretreatment step which indeed reduces the energy requirements to convert the stillage into biogas. Research and investigation is required to produce valuable by-products during the pretreatment process which could also maximize the cost-effectiveness of the process (Shirkavand et al., 2016). Therefore, development of mass and energy balance could help to take complete advantage of biomass energetic potential while reducing the costs (Cesaro and Belgiorno, 2015). Vast number of variables such as biomass type, interaction of the biomass with the pretreatment method, energy requirements, etc. are involved for the mass and energy balance. Recently, computational tools have been increasingly used for advancing the understanding of experimental pretreatment results and predicting the efficiency, economic viability and sustainability of the process. These machine learning approaches require large database to generate predictive models of biomass pretreatment efficacy and biofuel yield. State-of-the-art visualization technologies such as Raman spectroscopy, atomic force microscopy, fluorescent labeling could produce more data that would be useful to better understand the effect of pretreatment on the biomass (Zhou and Tian, 2022). Hence, reviews such as this article would help to fill the knowledge gap that exists to develop simulation tools and the synergy of computational and experimental studies help to develop full-scale viable lignocellulosic biomass conversion processes (Pham et al., 2022).

### **III.2.7 Conclusion**

Lignocellulosic biomass is a sustainable bioenergy source for the future. The choice of combined pretreatment strategies for LCB degradation depends on the downstream process. Combining two fungi or two enzymes did not improve the biogas yield because of slow degradation due to competition between the two fungi or excessive carbohydrate removal by two enzymes. Combining fungal pretreatment with alkaline pretreatment, improved the biogas yield. But combination of enzyme with alkaline pretreatment was better as fungal growth uses some nutrients that are essential for microorganisms in the AD process. Only alkaline pretreatment is studied so far for biological-chemical combined pretreatment for biogas production. Nevertheless, combination of biological with other chemical methods seem to provide a vast scope for research and process development for biogas production. Ethanol yield varies depending on the fermentation strategy and substrate consistency, when the same type of combined pretreatment is applied. Low substrate consistency leads to diauxic shift while high substrate consistency leads to inhibited enzyme action. When there is significant delignification, SSF is effective while for biomass with higher lignin content, SHF is effective.



For bioethanol production, fungal combined with organosolv appears to be the most studied process. Currently, strategies to co-ferment *S.cerevisiae* with other strains that utilize pentose sugars are being developed to obtain higher yields of ethanol. Using metabolic engineering to develop strains that are applicable in a wide range of environment for biofuel production is also an emerging field of study. Use of bacteria for LCB degradation is interesting as they can be easily engineered and because of their ability to exist in various environmental conditions. Currently, bacteria are increasingly used for ligninolytic enzyme production as the extent of delignification using bacteria has not reached the efficiency of white rot fungi. Bioaugmentation with anaerobic fungi for biogas production and genetic engineering of ethanol fermenting strains are emerging fields to improve biofuel yields.

## III.3 Lignin Fungal Depolymerization: From Substrate Characterization to Oligomers Valorization

### Lignin Fungal Depolymerization: From Substrate Characterization to Oligomers Valorization



Shruthi Meenakshisundaram, Estelle Léonard, Claire Ceballos,  
and Antoine Fayeulle

**Abstract** Renewable resources obtained via sustainable methodologies provide an alternative to reduce the dependency on fossil resources. Lignin is the second most abundant class of macromolecules on earth. This complex biopolymer is the major source of phenolic compounds since its precursors are three types of phenylpropane units (or monolignols), i.e., coniferyl alcohol (G), sinapyl alcohol (S), and *p*-coumaryl alcohol (H). Large amounts of lignin are recovered from the pulping industry using various processes such as Kraft, sulfite, soda anthraquinone, etc., due to its undesirability and high solubility during the cooking process. More recently, lignin is also being recovered during the pretreatment steps (Hydrolysis, Ionic Liquid, Organosolv processes) in biorefinery processes. Based on the extraction procedure and type of biomass used, the molecular structure of lignin will vary and has to be characterized for valorization. Due to the complexity of the structure of lignin, a combination of complementary techniques is used to characterize lignin. Various analytical techniques such as spectroscopy, chemical degradation, thermal degradation, chromatography, and the methods used to visualize the physical structural changes in lignin that help to determine the qualitative and quantitative properties bring different information. Of the techniques elaborated, magnetic resonance and Gel Permeation Chromatography (GPC) are found to be proficient in the elucidation of these complex biopolymers. Aromatic compounds are the basic building blocks in lignin and therefore, their potential in various applications is quite high. However, lignin's recalcitrance makes it difficult to break it down for further applications and requires high temperatures and high pressure for the same. Therefore, fungal depolymerization is a more economical alternative to break down lignin without damaging the cellulose and hemicellulose. Lignin decaying fungi excrete a powerful enzyme cocktail that generates highly reactive mediators which in turn react with the polymer and trigger extracellular depolymerization. The resulting low molecular weight soluble molecules such as vanillin, syringaldehyde, syringic acid, etc. are valuable for further applications and thus the optimization of fungal lignin depolymerization and recovery of oligomers before their uptake by fungal cells are ongoing domains of research. Indeed, some of the generated oligomers can be used in the generation of other valuable products such as chemicals and materials through green chemistry. Lignin-derived phenols are also used in bioremediation strategies. Besides, the use of fungal lignin depolymerization has great valorization potential to produce renewable energies such as biomethane, biohydrogen, and bioethanol without generating products that may be inhibitory in the downstream process. Therefore, with the increasing lignin-centric biorefineries, there is strong interest for fungal lignin depolymerization in both valorization and application approaches.

**Keywords** Analytical techniques · Fungal ligninolytic enzymes · Green chemistry · Lignin extraction · Lignin valorization

S. Meenakshisundaram · E. Léonard · C. Ceballos · A. Fayeulle (✉)  
Université de technologie de Compiègne, ESCOM, TIMR (Integrated Transformations of Renewable Matter), Centre de Recherche Royallieu, CS 60 319, Compiègne Cedex, France  
e-mail: [antoine.fayeulle@utc.fr](mailto:antoine.fayeulle@utc.fr)

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329

A book chapter was published in the book titled *Fungal Biopolymers and Biocomposites: Prospects and Avenues* in 2022 and reprinted here with the editor's permission. The core of the text is not modified, but only an extract is presented and the references are provided in Chapter X. This extracted text helps to focus only on the objectives of this thesis. The industrial lignin production process and the various applications of oligomers discussed in the book chapter (not presented here) are beyond the scope of this study.

### **III.3.1 Introduction**

Lignin is a widespread biopolymer entering the composition of all terrestrial plant biomasses with high recalcitrance to biodegradation. Indeed, along with impermeabilization and rigidification of plant tissues, this high molecular weight and hyper-variable polymer protect other polysaccharides of cell walls, i.e., cellulose and hemicelluloses, from microbial enzymatic attacks. In order to obtain such molecules, plants cells excrete different phenyl propanol precursors called monolignols, which are then polymerized in apoplastic domains through a random radical-based polymerization process involving notably laccases (Hoegger et al., 2006) and peroxidases (Tobimatsu and Schuetz, 2019). These monolignols are mainly composed of p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which correspond respectively to H-unit, G-unit, and S-unit in the polymerized lignin structure (Kleinert and Barth, 2008).

This potential non-food natural source of biobased aromatics derivatives makes lignin attractive for the development of biorefinery processes (Mahmood et al., 2016). Indeed, large quantities of lignin are produced in nature with estimated amounts ranging from 5 to  $36 \times 10^8$  tons per year (Gellerstedt and Henriksson, 2008). Concerning the significance of industrial residues derived from this total resource, biomass refinery and pulp/paper industries produce yearly  $6.2 \times 10^7$  and  $5 \times 10^7$  tons respectively mostly under the form of technical lignin such as Kraft lignin, lignosulfonate, and soda lignin (Zakzeski et al., 2010). However, the recalcitrance of the lignin polymer to degradation complexifies also its valorization in industrial processes, and lignin residues are by now mostly burned for energy supply or wasted (Weng et al., 2021).

Existing lignin depolymerization techniques include thermochemical approaches such as pyrolysis or microwave treatment, and chemical protocols with acid-, base-, metallic, ionic liquid assisted, subcritical/supercritical fluids assisted, or oxidative catalysis. These techniques

lead to high lignin depolymerization rates but require severe conditions such as high pressure and temperature leading to high-energy consumption and the use of environmentally threatening substances. Thus, the third type of approach based on biocatalysts including enzymes or whole microorganisms aims at developing more eco-compatible and chemoselective lignin depolymerization bioprocesses (Chio et al., 2019).

Bacteria attracted interest in particular due to the pre-existence of industrial processes more adapted to bacterial cultivation (Xu et al., 2018). Ligninolytic bacteria described as far belong to *Sphingomonas*, *Pseudomonas*, *Rhodococcus*, *Nocardia*, and *Streptomyces* genera (Bugg et al., 2016) and in particular, *Rhodococcus jostii*, *Pseudomonas putida*, *Acinetobacter* sp. and *Amycolatopsis* sp. were investigated due to their depolymerization abilities of high molecular weight lignin molecules (Salvachúa et al., 2015). However, the lignin depolymerization ability is described as scarcer within the bacterial biodiversity, and the conversion efficiency is much lower than in fungi (Chio et al., 2019).

Thus, the goal of this chapter is to provide up-to-date information required for the development of lignin fungal depolymerization bioprocesses including natural and technical lignin properties, characterization techniques of lignin molecules, mechanisms involved in fungal lignin depolymerization, and main valorization ways for the so produced molecules.

### **III.3.2 Analytical Methods Adapted to Lignin Characteristics**

In the 1960–1970s, prominent structures in softwood and hardwood lignin were proposed by Adler (1977). Since then, many models of each linkage and lignin unit type in literature were built. The difficulties in lignin analysis reside in its complex structure associated with plant cell wall hemicelluloses (Sjöström, 1993) which interfere with the quantification of lignin (Brunow and Lundquist, 2010). The acknowledgment concerning the structure of lignin is essential to understand the properties of lignin and the effect of pretreatment. There is no universal method to characterize the lignin but a combination of complementary techniques to provide insights into the structure of the polymer. Some reviews compiled recent innovations in studying lignin (Ghaffar and Fan, 2013; Lupoi et al., 2015).

Most of the characterization methods of lignin are summarized in Table III-5. The advantages or disadvantages of these techniques are stated. The composition of lignin, its structure and linkages, and destructive analysis techniques are presented. Spectroscopic techniques, thermal and physical methods will be then described. Finally, the last part will be

devoted to steric exclusion chromatography, one of the essential techniques for the study of soluble lignin.

**Table III-5:** Main methods for lignin analysis

Methods	Techniques	Information	Advantages	Disadvantages
Chemical degradation	Thioacidolysis, DFRC, nitrobenzene oxidation	S/G ratio, monolignols quantification	Well-standardized techniques	Tedious, Destroys the sample, Toxicity
Spectroscopic methods	FTIR	Functional groups	Non-destructive, High throughput	Non-destructive, Non-invasive, Sensible to water
	Raman	Functional groups	Complimentary to IR, Non-destructive	Weak pics, fluorescence masks signal
	UV	Phenol content	Cheap, convenient	The extinction coefficient for lignin quantification difficult to obtain Absorbance in UV of extraneous molecules
	NMR	Aliphatic hydroxyl content, Aromatic hydroxyl content, Inter-unit bonding patterns, S/G ratio	Multi-dimensional NMR, Many structural information	Spectral overlap, Low throughput, Low sensitivity, spectral resolution, Expensive, Incomplete acetylation of lignin leading to not complete NMR
Physical properties	Optical microscopy	Morphology	Fast analysis,	Expensive apparatus,

	SEM	Morphology, size, crystalline structure	Non-destructive	No structural information
	TEM	Crystalline nature		
	X-ray	Ultrastructure, fiber diameter		
	FE-SEM	Morphology, surface changes		
	AFM	Morphology		
Molecular structure	SEC	Molecular Distribution, Weight average molar mass, Polydispersity	Large detectable masses	Condensation effects, Measurement can vary following the technique
Chromatography	GC-MS, oxidation	S/G ratio, Phenolic content	Selective	Results depend on condensed structures in lignin, Destructive, Concern volatiles molecules
	Aminolysis	Phenolic content, Aliphatic hydroxyl content		

### III.3.2.1 General Composition of Lignin

Lignin as well as its different impurities such as ash, sugar, and moisture are determined by the different standards such as ISO, ASTM, or TAPPI (as shown in Table III-6). The total lignin content is determined according to the NERL/TP-51042618 protocol (Sluiter et al., 2012) and is considered as the standard method for quantitative lignin analysis. According to this protocol, the sample is hydrolyzed with concentrated H<sub>2</sub>SO<sub>4</sub> (72%) then diluted to obtain an acid concentration of 3%. The polymeric carbohydrates are degraded in monosaccharides, leaving behind a lignin-rich residue, named Klason lignin or acid-insoluble lignin (AIL), that is vacuum filtered and measured gravimetrically (Gosselink et al., 2004b; Vishtal and Kraslawski, 2011).

**Table III-6:** Determination of the general composition of Kraft lignin

	<b>Technique</b>	<b>Notes</b>
Total lignin content Klason lignin	Gravimetry (Tappi T222 om-15)	0–30% wt lignin content for wood and pulp. Overestimation of the lignin which condenses with proteins or chitins
Acid soluble lignin	UV-Vis Spectroscopy (Tappi UM-250)	A reliable method for wood and pulp. Total lignin corresponds to insoluble and soluble lignin
Van Soest, acid detergent lignin (ADL)	Gravimetry (Hatfield et al., 1994; P.J. Van Soest and Wine, 1968)	An alternative procedure to Klason lignin and replaces the use of corrosive sulfuric acid by permanganate oxidation to remove protein, hemicelluloses, and other components from the cellulose and lignin
Ash content	Ashing Tappi method T211 om-12	Several temperatures (525–950°C) used
Carbohydrates	Ion chromatography (SCAN-CM 71) Spectroscopy UV	Measure free or total sugars
Metals	Inductively coupled plasma atomic emission spectroscopy (ICP-AES)	Al, Cu, Fe, Mg, Ca, K...
Sulfur ions	Ion chromatography for sulfur (SCAN-CM 57), for sulfate (ISO-9198)	0–10% wt
Moisture	Gravimetry	

### **III.3.2.1.1 Elemental Analysis**

The elemental analysis gives access to the various elemental compositions of compounds (including lignin) in terms of carbon, hydrogen, nitrogen, or sulfur. These compositions can sometimes give access to the formula of the tested compounds. The formula is expressed in terms of the element content of the average phenylpropane C9 unit present in the lignin (Norman, 1969). The amount of methoxy function is also often incorporated into this formula, for example,  $C_9H_{8.83}O_{2.37}(OCH_3)_{0.96}$  for spruce in milled wood lignin (Huang et al., 2019). The elemental composition of lignin depends on the sources of raw material and separation methods (Huang et al., 2019). The highest levels of sulfur are observed in the case of lignosulfonates (Svensson, 2008).

### **III.3.2.2 Spectroscopic Methods**

Spectroscopic methods particularly ultraviolet and infrared spectroscopic techniques can be used without treatment of lignin (Gilarranz et al., 2001). Infrared spectroscopy has become a routine technique for the analysis of lignin based on the spectral identity of different types of lignin (Derkacheva and Sukhov, 2008; Faix and Beinhoff, 1988). NMR is also a very powerful tool for the analysis of native lignin (Ralph et al., 2007).

#### **III.3.2.2.1 Fourier Transform Infrared Spectroscopy**

FTIR is a non-destructive spectroscopic technique giving information on functional groups of organic compounds. Infrared is often used in the characterization of lignin due to some advantages: short analysis time, high sensitivity, easy use, direct analysis on solid samples, and convenient data handling (Faix, 1991). Lignin contains various functional groups: carbonyl, methoxyl, hydroxyl, and carboxyl groups, aromatics (Fengel and Wegener, 1983), and their vibrational bands are summarized in Table III-7. The region between 1400 and 1800  $cm^{-1}$  corresponds to carbonyl functions such as the elongation vibrations of non-conjugated and conjugated carbonyl bonds (respectively at 1709 – 1738 and 1655 – 1675  $cm^{-1}$ ). Their intensity and shift reveal (Faix, 1991; Hortling et al., 1997) the efficiency of derivatization reactions and analyze different types of lignin. For example, the study of the infrared spectra from technical lignin by El Mansouri and Salvadó (2007) highlighted clear differences between these lignin (Figure III-2) but this method is not quantitative.



**Table III-7:** Assignments of FT-IR absorption bands ( $\text{cm}^{-1}$ )

<b>Absorption bands</b>	<b>Assignment</b>
3400 – 3600	Free OH stretching
3100 – 3400	Associated OH
2820 – 2960	CH stretching of CH, CH <sub>2</sub> , CH <sub>3</sub> group
2920	CH stretching of carboxylic OH
2650 – 2890	CH of CH <sub>3</sub> group in methoxyl
1771	CO aromatic
1700 – 1750	C=O stretch in unconjugated ketone, carboxyl group
1722	C=O stretch aliphatic
1650 – 1680	C=O stretch in conjugated ketone
1500 – 1600	Aromatic skeletal vibration
1420 – 1430	Aromatic skeletal vibration
1460	Aromatic methyl group vibrations
1434	Aromatic skeletal vibration
1374	Aliphatic C–H stretch in CH <sub>3</sub>
1328	Syringyl ring with C–O stretching
1270 – 1275	C–O Guaiacyl ring
1242	Aromatic C–O stretching
1215	C–O ether stretching
1165	C–O stretch in ester groups
1135	Aromatic C–H in-plane deformation for syringyl type
1043	Aromatic C–H in-plane deformation for guaiacyl type
750 – 860	Aromatic C–H out of plane bending

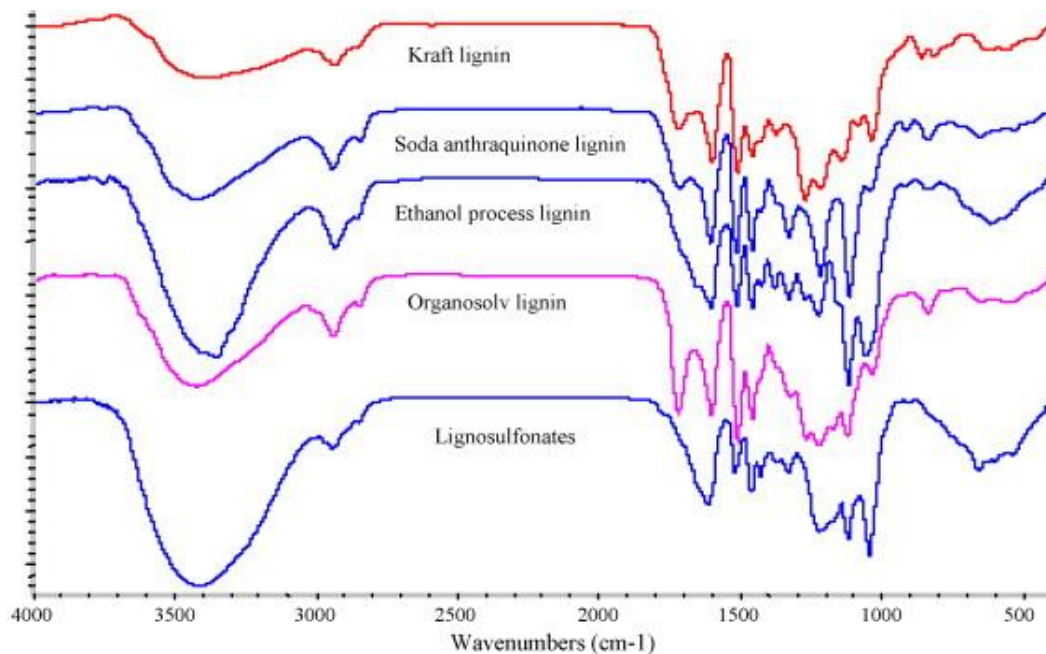


Figure III-2: FTIR spectra of unacetylated lignin samples. (Reuse (print) with permission of El Mansouri and Salvadó (2007). Analytical methods for determining functional groups in various technical lignin. *Industrial crops and products*. 26, 116–124. <https://doi.org/10.1016/j.indcrop.2007.02.006>. Copyright (2007) Elsevier)

### III.3.2.2.2 Raman

Raman is vibrational scattering spectroscopy complementing IR. Raman photons are emitted when a sample is illuminated by a laser source (Near IR-Visible and UV) through the phenomenon of inelastic light scattering. Like FTIR, no sample preparation is required and Raman takes short analysis times (similar to FTIR). Several Raman scattering peaks can be attributed to lignin at  $1600\text{ cm}^{-1}$  (aromatic ring stretch), at  $1620\text{ cm}^{-1}$  (carbonyl content),  $1650\text{ cm}^{-1}$ , and at  $3070\text{ cm}^{-1}$  (aromatic C–H stretch) (Agarwal, 2008; Agarwal et al., 2011; Larsen and Barsberg, 2010). The intensities of the peak at  $1650\text{ cm}^{-1}$  (C=C) relative to those at  $1600\text{ cm}^{-1}$  (phenol ring) may explain the degradation of the lignin (Moosavinejad et al., 2019). Raman spectroscopy allows the measurement of S/G ratios in diverse feedstocks (Lupoi et al., 2015). The major drawback of this technique is that Raman peaks are often of weak intensities and can be completely masked by the fluorescence of lignin. It is however noticeable that the surface-enhanced Raman scattering (SERS) enhances the signal by molecules adsorbed on rough metals surfaces (Agarwal, 1999).

### III.3.2.2.3 UV-Spectrometry

Lignin aromatic structure exhibits maximal absorption in the ultraviolet region around 280 nm and at 315 nm if ester groups are present such as in grass lignin. The absorbance difference before and after ionization of phenolic groups in alkaline solution at a wavelength of 300 and 350 nm has been used to measure the content of phenolic hydroxyl groups (Goldmann et al., 2016).

Figure III-3 illustrates differences in UV spectroscopy between LignoBoost and alkali-treated lignin samples (Abdelaziz and Hulteberg, 2017).

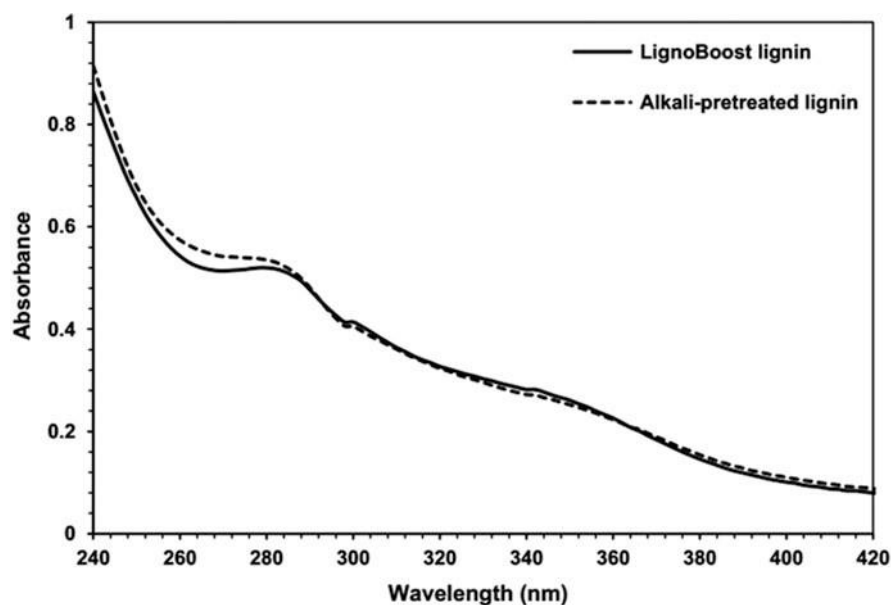


Figure III-3: UV-Visible spectra of lignin obtained from the LignoBoost and alkali pretreatments. (Reuse (print) with permission of Abdelaziz and Hulteberg (2017). Physicochemical Characterisation of Technical Lignins for Their Potential Valorisation. Waste Biomass Valor 8, 859–869. Copyright (2017) Springer)

### III.3.2.3 Nuclear Magnetic Resonance

Since the 1970s, NMR analysis of lignin has continuously evolved (Ludwig et al., 1964; Lundquist, 1992). NMR spectroscopy is a highly sensitive tool to characterize lignin. The most commonly used NMR probes are  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$ . However, despite the ease of use, drawbacks remain, as many lignin need acetylation before analysis, because they are insoluble in common deuterated NMR solvents. In this context, Lundquist's research group reported the first  $^1\text{H}$  NMR to analyze the milled wood lignin (Li and Lundquist, 1994; Lundquist et al., 1980, 1979a,

1979b). The  $^1\text{H}$  NMR of acetylated lignin in  $\text{CDCl}_3$ , as seen in Figure III-4, allows to identify methyl protons in the aliphatic acetyl groups (1.70 – 2.17 ppm), protons in methoxyl groups (3.5 – 4.0 ppm), aliphatic protons in the linkages of  $\beta\text{-O-4}$ ,  $\beta\text{-}\beta$ , and  $\beta\text{-5}$  (4.0 – 6.3 ppm), and aromatic protons (6 – 7.10 ppm) (Chu et al., 2013; Mancera et al., 2011). Anyway, as seen in Figure III-4 (Tejado et al., 2007), lignin analysis by  $^1\text{H}$  NMR of lignin remains complex due to the significant superposition of several characteristic resonances (Lundquist, 1992), making the proton spectra of lignin not really exploitable. The wider spectral window (200 ppm) of carbon NMR allows better discrimination of resonances, thus allowing the identification of the structural motifs of lignin.

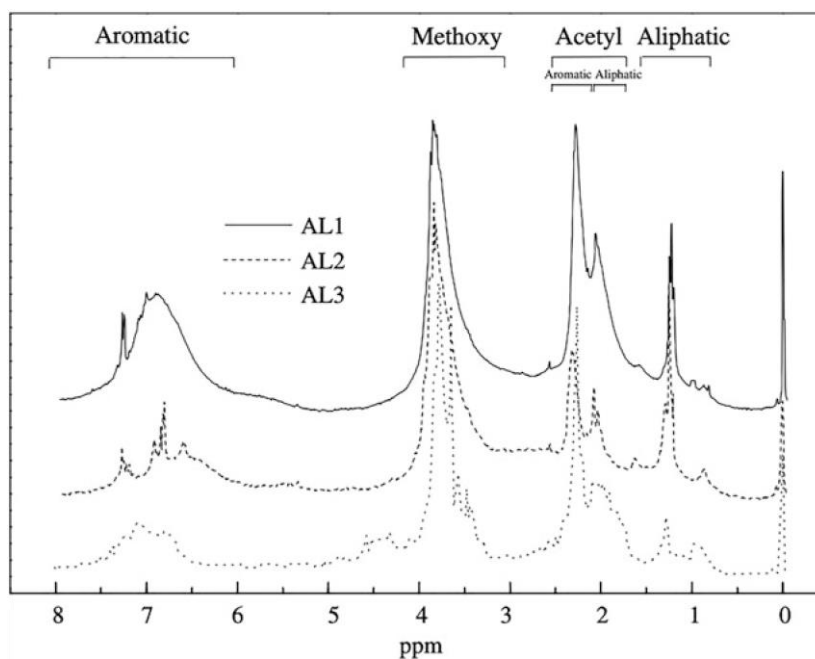


Figure III-4:  $^1\text{H}$  NMR spectra of acetylated Pine (AL1), Flax (AL2), and Tamarind lignin (AL3) samples. (Reuse (print) with permission of Tejado et al. (2007). Physico-chemical characterization of lignin from different sources for use in phenol-formaldehyde resin synthesis. *Bioresource Technology* 98, 1655–1663. <https://doi.org/10.1016/j.biortech.2006.05.042> Copyright (2007) Elsevier)

The spectroscopic technique of  $^{13}\text{C}$  NMR is now widely employed for the study of lignin.  $^{13}\text{C}$  NMR spectroscopy of lignin allows the assignment to the various carbons of the lignin skeleton including the presence of aryl ether, condensed and uncondensed aromatic, and aliphatic carbons. The area between 60 and 80 ppm is attributed to the oxygenated aliphatic carbons (carbons carrying the alcohol functions for example), the signals of the aromatic

carbons come out between 100 and 160 ppm and finally, the zone between 160 and 180 ppm corresponds to the carbons of carboxylic acids. The attributions are made by comparison with the numerous existing databases (Chen and Robert, 1988; Kringstad and Mörck, 1983), which were carried out thanks to the analysis of models. For example, the structural features of the lignin fractions from *Eucalyptus pellita* were investigated with  $^{13}\text{C}$  NMR spectrometry, Figure III-5 (Yuan et al., 2009). The  $^{13}\text{C}$  NMR spectroscopy requires a large sample size and a long acquisition time. To overcome the problems of solubility or modifications of lignin, solid-state cross polarization-magic angle spinning  $^{13}\text{C}$  NMR is an alternative (Cipriano et al., 2020). In this technique, the sample is spinning very rapidly (up to  $\sim 100,000$  rpm) at a precise angle, the magic angle ( $\theta_m \approx 54.7^\circ$ ), to improve the resolution of the spectrum.

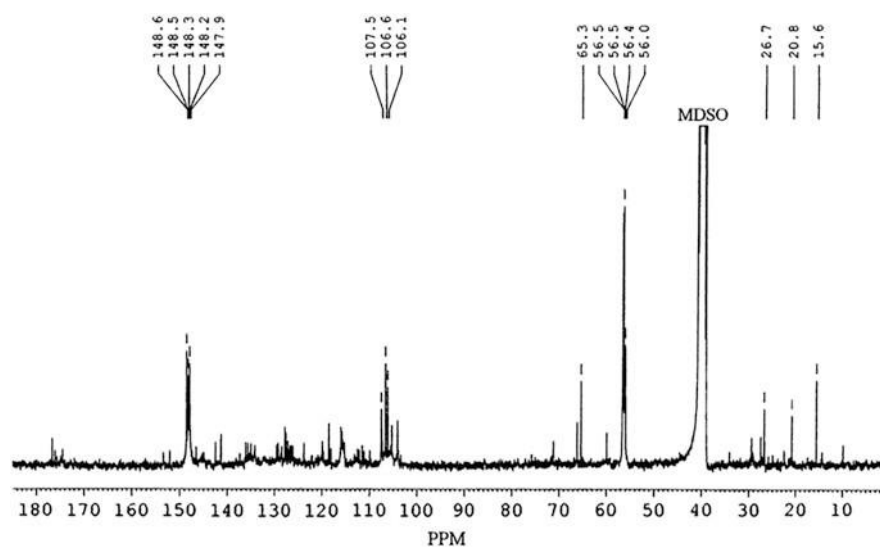


Figure III-5:  $^{13}\text{C}$  NMR spectrum of the degraded lignin fraction. (Reuse (print) with permission of Yuan et al. (2009). Fractionation and physico-chemical analysis of degraded lignin from the black liquor of *Eucalyptus pellita* KP-AQ pulping. *Polymer Degradation and Stability* 94, 1142–1150. <https://doi.org/10.1016/j.polymdegradstab.2009.03.019>. Copyright (2019) Elsevier)

$^{31}\text{P}$  NMR can be used as a complementary analysis to  $^{13}\text{C}$  NMR. Phosphorylation of the hydroxyls carrying labile protons of lignin with 2-chloro-4,4,5,5-tetramethyl 1,2,3-dioxaphospholane (TMDP) in anhydrous pyridine/ $\text{CDCl}_3$  (1.6/1 v/v) solution give phosphite products (Argyropoulos, 1994; Pu et al., 2011). For example, a typical  $^{31}\text{P}$  NMR spectrum of an organosolv lignin isolated from bamboo is shown in Figure III-6. Total aliphatic hydroxyl, phenolic hydroxyl, and carboxyl groups, as well as the G/S/H ratio of lignin samples, are

established by quantitative  $^{31}\text{P}$  NMR using published procedures (Cateto et al., 2008; Faix et al., 1994). Anyway,  $^{31}\text{P}$  NMR analysis requires intensive sample preparation.

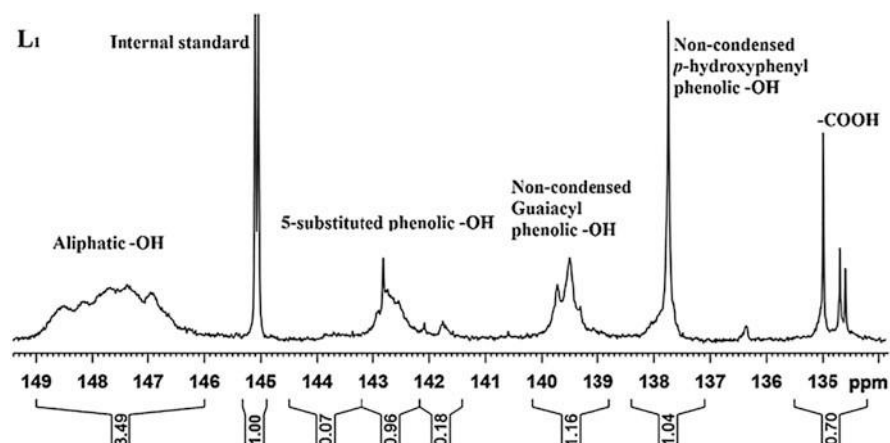


Figure III-6:  $^{31}\text{P}$  NMR spectrum of organosolv lignin isolated from bamboo. (Reuse (print) with permission of Liao et al. (2020). Current advancement on the isolation, characterization and application of lignin. *International Journal of Biological Macromolecules*, 162, pp. 985–1024. <https://doi.org/10.1016/j.ijbiomac.2020.06.168>. Copyright (2020) Elsevier)

Interpretation  $^1\text{D}$  NMR (one-dimensional NMR) is based on 3000 structures of lignin and derivatives (from monomers to tetramers) database (Ralph et al., 2004) but is limited to simple patterns or specific band assignments. Thus, researchers turn to two-dimensional NMR techniques to explore more structural elucidation and the presence of some particular structures, in low abundances such as spirodienone.

The two-dimensional measurements (heteronuclear single quantum coherence, HSQC; heteronuclear multiple quantum coherence, HMQC) combine both  $^{13}\text{C}$  and  $^1\text{H}$  NMR. As illustrated, the identification of different substructures present in lignin polymer is achieved through HSQC as displayed in Figure III-7 (Ibarra et al., 2007; Liitiä et al., 2003; Ralph et al., 2004; Rencoret et al., 2008). Database enrichment allows confirmation of correlation assignment on HSQC and HMQC spectra of native lignin (Ibarra et al., 2007; Liitiä et al., 2003; Ralph et al., 2004) and also determines the nature of different bonds and structures present in lignin.

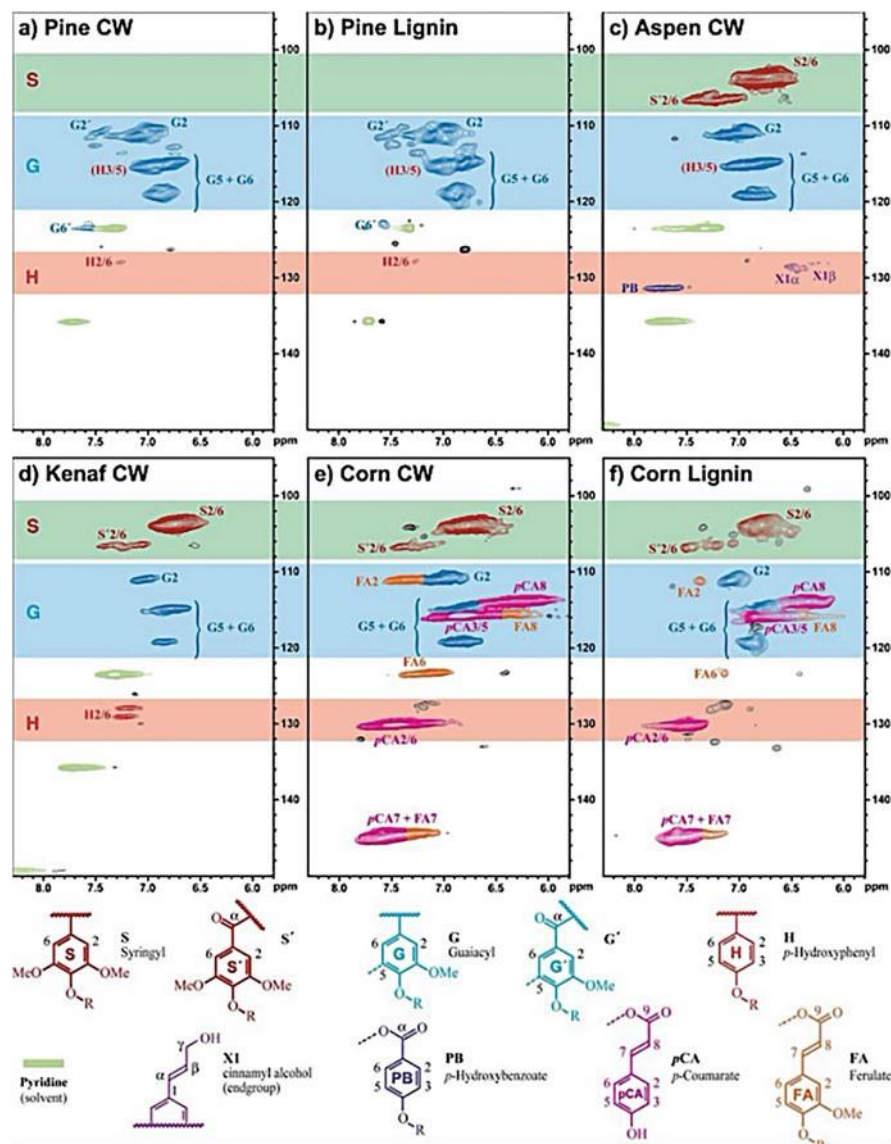


Figure III-7: Aromatic regions of 2D-HSQC NMR spectrum from cell wall gels and soluble lignin from various samples: (a) pine, (b) pine isolated lignin, (c) aspen, (d) kenaf bast fiber, (e) corn stems, and (f) corn isolated lignin. (Reuse (print) with permission of Liao et al. (2020). Current advancement on the isolation, characterization and application of lignin. International Journal of Biological Macromolecules, 162, pp. 985–1024. <https://doi.org/10.1016/j.ijbiomac.2020.06.168>. Copyright (2020) Elsevier)

### III.3.2.4 Thermal Analysis

Thermal analyses reveal the structure-properties relationships of polymers as a function of temperature. Thermogravimetric analysis (TGA) and differential enthalpy analysis (DSC)

are widely used to study the thermal behavior of lignin (Poletto, 2017). TGA allows monitoring the lignin weight loss when a sample is heated, cooled, or maintained at a fixed temperature. For most lignins, three stages of mass loss are observed when the analysis is performed under inert atmosphere (N<sub>2</sub>): drying (20 – 150°C), pyrolysis (200 – 500°C), and carbonization (500 – 900°C). DSC is a thermo-analytical method where the temperature of a sample and reference material is increased at a constant rate and allows to determine the glass transition temperature (T<sub>g</sub>) and the enthalpy of lignin due to changes in the lignin polymer (Figure III-8). For example, lignin T<sub>g</sub> determined by DSC is usually comprised between 110 and 160°C for milled wood lignin and 124–174°C for Kraft lignins (Glasser et al., 1999) but is influenced by several factors such as the aromatic structure, hydrogen bonding type interactions, the molar mass, the type of inter-unit bonds and the degree of cross-linking (Köhnke et al., 2018). Comparison of the glass transition temperatures (T<sub>g</sub>) of different modified lignins are reported (Ding et al., 2016).

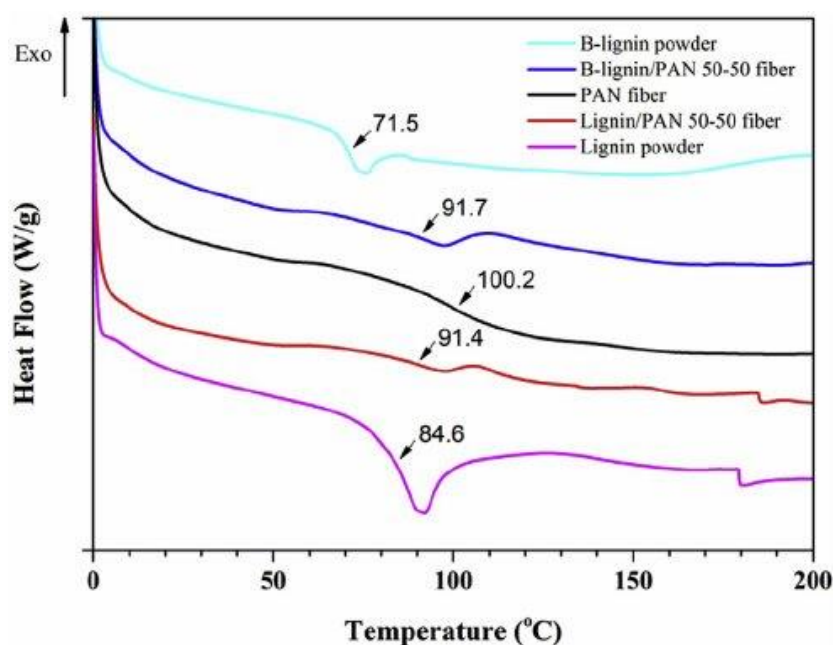


Figure III-8: Glass transition temperature (T<sub>g</sub>) of lignin, B-lignin (Butyrate organosolv lignin), and precursor fibers based on PAN (polyacrylonitrile), lignin/PAN 50–50, and B-lignin/PAN 50–50 measured by DSC. (Reuse (print) with permission of Ding et al. (2016). Processing and Characterization of Low-Cost Electrospun Carbon Fibers from Organosolv Lignin/Polyacrylonitrile Blends. Carbon 100. <https://doi.org/10.1016/j.carbon.2015.12.078>. Copyright (2015) American Chemical Society)



### III.3.2.5 Pyr/Gas-Chromatography MS

Analytical pyrolysis is an efficient technique for the analysis of the chemical composition of wood and in particular for the characterization of lignin of various origins. Pyrolysis/gas chromatography-mass spectrometry (Pyr/GC-MS) is very interesting to evaluate the ratios S/G in lignin (Lopes et al., 2011). Pyrolysis consists of heating a sample to a high temperature ( $>500^{\circ}\text{C}$ ) in an inert atmosphere to cleave the constituent polymers into small fragments. These more volatile products formed flushed by helium can be separated by chromatography generally coated with an apolar silicone phase. Peaks of the pyrolysis products on GC give a specific pyrogram (Figure III-9) whose peaks can be identified according to their mass spectra obtained by electronic impact (70 eV) (Galletti and Bocchini, 1995).

Studies confirmed the ability of the Py/GC-MS technique to provide useful data for the characterization of lignin (Lima et al., 2008; Lucejko et al., 2020). Whereas being a sensitive analysis used for the examination of lignin, Py/GC-MS presents major drawbacks: greater expense instrumentation, long analysis times (typically between 40 min and 2 h per sample), and complex interpretation of data.

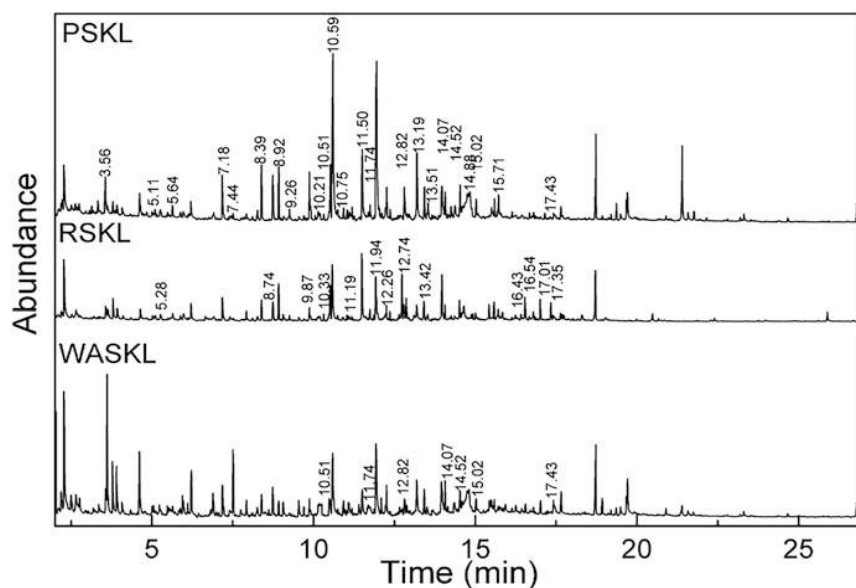


Figure III-9: Typical ion chromatograms from pyrolysis of the G, GS, and HGS type lignin from variant biomass resources peanut shell walnut shell (PSKL), rape straw (RS), wheat straw (WASKL). (Reuse (print) with permission of Chen et al. (2015). Study on pyrolysis behaviors of non-woody lignins with TG-FTIR and Py-GC/MS. Journal of Analytical and Applied Pyrolysis 113, 499–507. <https://doi.org/10.1016/j.jaap.2015.03.018>. Copyright (2015) Elsevier)

### III.3.2.6 Physical Qualitative Properties

Optical microscopy is an easy method to observe morphological aspects, the particle size of biomass, and the lignin distribution, for example within straw. Scanning electron microscopy (SEM) is used to observe its morphology and size (Mandlekar, 2019), and small-angle or ultra-small-angle X-ray scattering can be used to study the supramolecular structure of lignin in both solid-state and in solution. Fromm et al. (2003) reported also the distribution of lignin in wood cell walls by field emission scanning electron microscopy (FE-SEM) (Figure III-10), which is a technique used for qualitative analysis of biomass. Transmission electron microscopy is also useful to analyze lignin nanoparticles allowing a clear contrast between the core and the shell. Atomic Force Microscope (AFM) is another method that enables evaluate surface structures of different lignin at nanometer scales.

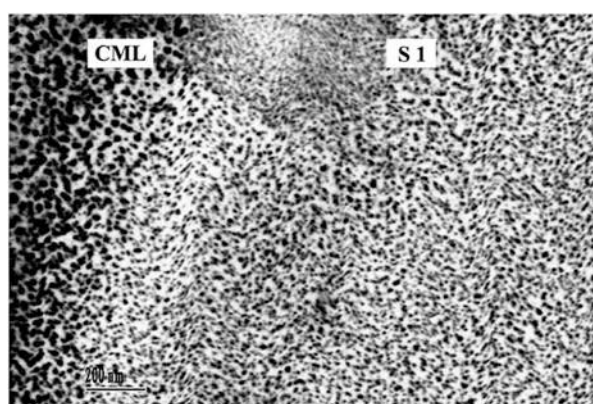


Figure III-10: FE-SEM micrograph. The transverse section shows lamellae of bright cellulose microfibrils and dark lignin aggregates. CML compound middle lamella, S1 secondary wall 1. (Reuse (print) with permission of Fromm et al. (2003). Lignin distribution in wood cell walls determined by TEM and backscattered SEM techniques. *Journal of Structural Biology* 143, 77–84. [https://doi.org/10.1016/S1047-8477\(03\)00119-9](https://doi.org/10.1016/S1047-8477(03)00119-9). Copyright (2003) Elsevier)

### III.3.2.7 Molecular Structure

Molar mass distribution of lignin is important to understand the fundamental properties of lignin and to know their exact molecular weight (Gellerstedt, 1992). The determination of molecular weight distribution is usually performed by steric exclusion chromatography (SEC) or also called gel permeation chromatography (GPC) when an organic solvent is used as a mobile phase. Dissolved molecules are eluted through a specific column filled with a nanoporous gel (stationary phase) and separated by size. Recent work by Baumberger et al.

(2007) focused on standardizing the SEC method for lignin analysis. The molar mass of acetylated Kraft lignin obtained from various black liquors varies mainly in the range of 200 – 15,000 g/mol. To improve their solubility, acetylation of lignin is sometimes done (Asikkala et al., 2012). In most cases, polystyrene gel with THF is used to calibrate molar mass distribution for different lignins (Baumberger et al., 2007). GPC spectra of soda lignin (SL) extracted with organic solvents are displayed for example in Figure III-11. Results exhibited that soda lignin (SL) was sequentially fractionated by organic solvents (ethyl acetate: F1, methanol: F2, acetone: F3, dioxane/water: F4) (Kim et al., 2017).

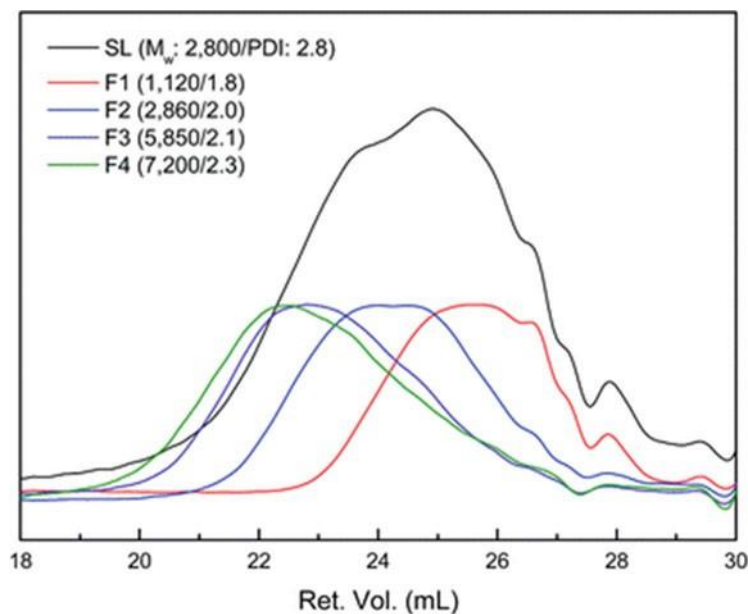


Figure III-11: GPC curves of lignin fractions (the peak maxima were normalized to same value except for SL) (Kim et al. 2017). (Reuse (print) with permission of Kim et al. (2017). Sequential solvent fractionation of lignin for the selective production of monoaromatics by Ru catalyzed ethanolysis. RSC Adv. 7, 53117–53125. <https://doi.org/10.1039/C7RA11541E>. Copyright (2017) Royal Society of Chemistry)

### III.3.2.8 Lignin Functional Groups-Based Approaches

Lignin has various functional groups such as phenolic hydroxyl, aliphatic hydroxyl, benzyl alcohol, noncyclic benzyl ether, carbonyl groups, carboxylic acids, and methoxyl groups (Figure III-12). The abundance of these functional groups directly affects the reactivity of the lignin in various chemical reactions. The use of selective reagents is often needed to analyze lignin functional groups and Table III-8 summarizes their determination methods (El Mansouri and Salvadó, 2007; Gosselink et al., 2004a).

To summarize, the synergistic combination of many methods each bringing partial but complimentary information is compulsory to understand the complex and heterogeneous lignin. Due to its extreme heterogeneity, the nature of lignin carbohydrate linkages and occurrence is not completely resolved. Many studies and techniques were led until now as SEM, SEC, FT-IR, TGA/DSC, NMR, and DFRC. But, the problem inherent in the structural investigation of lignin is still important. Nevertheless, the development of new methodologies has improved our understanding of the complex structure of these polymers. In particular, the magnetic resonance techniques and GPC have proved to be efficient analytical tools for the structural elucidation of these complex biopolymers.

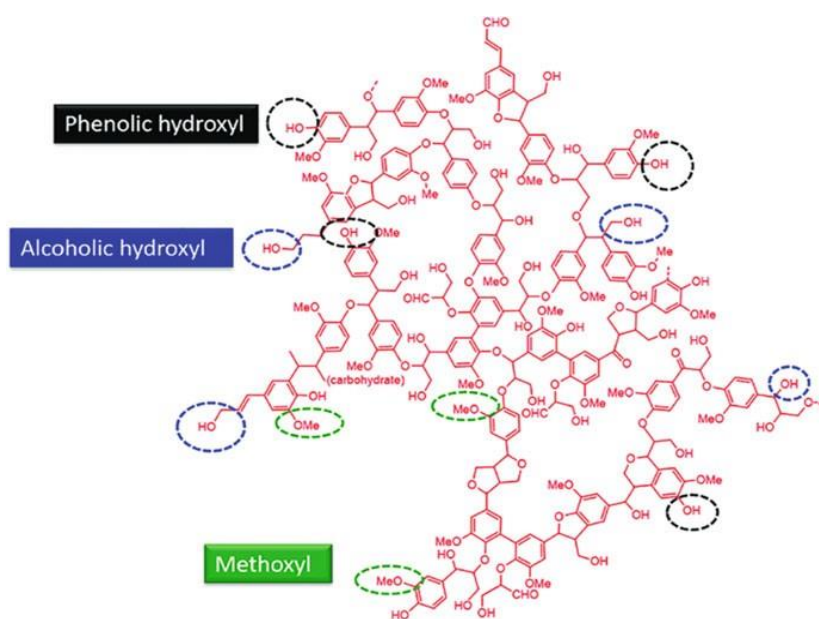


Figure III-12: Main functional groups of lignin. (Reuse (print) with permission of Poursorkhabi et al. (2020). Processing, Carbonization, and Characterization of Lignin Based Electrospun Carbon Fibers: A Review. *Frontiers in Energy Research* 8, 208. <https://doi.org/10.3389/fenrg.2020.00208>. Copyright (2017) National Renewable Energy Laboratory (DOE))

**Table III-8:** Main analytical methods to characterize the functional groups of lignin

Functional groups	Technique
Phenolic and Aliphatic Hydroxyl groups	Aminolysis
	UV visible spectrometry
	Potentiometric titration
	Acetylation/quantitative $^{13}\text{C}$ NMR
	Periodate oxidation
	$^1\text{H}$ NMR/ $^{31}\text{P}$ NMR methods
Benzyl Ether and Benzyl Alcohol groups	Acid hydrolysis in methanol
	NMR
Carbonyl groups	Hydroxylamine hydrochloride method
	$\text{NaBH}_4$
Carboxylic acid groups	Conductimetry with titration
	– Titration in the aqueous phase
	– Titration in non-aqueous phase by tetra-n-butylammonium hydroxide (TnBAH)
Methoxyl content	Gaz chromatography by indirect measurement

### III.3.3 Fungal Enzymes and Mechanisms Involved During Lignin Depolymerization

Due to the recalcitrance of lignin to biodegradation, the ability to depolymerize it and to use the obtained oligomers as sources of carbon and energy is a true evolutionary advantage and represents a key activity for the carbon cycle in the ecosystems. Fungi are reported as the most efficient microorganisms for lignin degradation (Chio et al., 2019; Weng et al., 2021) and developed the capacity to produce ligninolytic enzymes in relation to their ecological role as wood degraders, soil litter-decomposers, or pathogens (Choi et al., 2014; Hofrichter, 2002). The use of these natural fungal activities to depolymerize lignin appears attractive for the

development of new bioprocesses with a reduced environmental impact compared to the physicochemical approaches. Thus, the functioning of the cellular and enzymatic mechanisms involved in lignin biodegradation was widely investigated in the past and is still the object of active research nowadays.

### III.3.3.1 Lignin Depolymerizing Enzymes from Fungi

Lignin decaying fungi excrete enzymes able to depolymerize the high molecular weight and hyper-variable molecule of lignin. Indeed, these enzymes do not directly interact with lignin but generate highly reactive oxidizing mediators able to chemically react with this polymer and trigger degradation ranging from its partial depolymerization to mineralization in CO<sub>2</sub> and H<sub>2</sub>O (Li and Zheng, 2020). These enzymes belong either to phenol oxidases with laccases or to heme peroxidases with lignin-, manganese-, versatile and dye decolorizing peroxidases (Table III-9). Along with these main enzyme types, the role of polyphenol oxidases was also described for lignin degradation by some fungi such as *Lentinula edodes* and *Pleurotus ostreatus* (Dong et al., 2013). Other enzymes such as aromatic peroxygenases have also been suggested to be involved in the conversion of lignin oligomers (Hofrichter et al., 2010). The oxidative reactions involved in lignin decomposition and catalyzed by this mixture of oxidoreductases include the cleavage of C – C bonds and ether linkages as well as the removal of side chains and aromatic rings (Zabel and Morrell, 2020).

Laccases are copper-containing enzymes that use molecular oxygen as a final electron acceptor for the cleavage of aromatic rings (Munk et al., 2015) or for the oxidation of phenolic substrates to phenoxy-radicals, which can then react with the lignin molecule (Gianfreda et al., 1999) and promote C $\alpha$  oxidation, alkyl-aryl cleavage, and C $\alpha$  – C $\beta$  cleavage (Kawai et al., 1988). Consequently, laccases are reported to trigger various lignin modifications such as polymerization, depolymerization, C $\alpha$  oxidation, and demethylation. Laccases can also oxidize non-phenolic compounds with the help of mediators such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3-hydroxyanthranilate (HAA), or 1-hydroxybenzotriazole (HBT) which stabilize the reaction intermediates (Hilgers et al., 2018). The oxidized non-phenolic compounds coupled with these mediators can promote the aromatic ring cleavage, C $\alpha$  – C $\beta$  cleavage, C $\alpha$  oxidation, and  $\beta$  ether cleavage (Kawai et al., 2004). Thus, with these kinds of mediators, laccases can degrade up to 90% of the lignin structure (Camarero et al., 2004).

Lignin peroxidases (LiP) are glycoproteins (38–43 kDa) using hydrogen peroxide generated by extracellular oxidases such as glyoxal or aryl-alcohol oxidases to perform one-electron oxidation of different phenolic and non-phenolic structures related to lignin (Weng et al., 2021), resulting in the formation of aryl cation radicals. These reactive cationic radicals are in turn capable of chemically attacking the lignin polymer (Pelmont, 1995). The catalytic cycle uses in particular veratryl alcohol (VA) as an electron donor and cofactor (Wong, 2009). LiPs are considered as the most efficient peroxidase type because it has a high redox potential enabling it to oxidize a higher variety of substrates (Sigoillot et al., 2012). Thus, LiPs are mostly described as the major enzyme responsible for lignin depolymerization and degradation (Weng et al., 2021). However, some studies hypothesized this kind of enzyme to have more of a role of detoxification of low molecular weight phenolic compounds released during lignin depolymerization or of functionalization of the lignin polymer to facilitate its depolymerization by other enzymes (Sarkanen et al., 1991).

Manganese peroxidases (MnP) are glycosylated heme enzymes very similar to LiP, but they use hydrogen peroxide to oxidize  $Mn^{2+}$  in the presence of chelators to  $Mn^{3+}$ , which in turn oxidizes a variety of phenolic substrates. In particular,  $Mn^{3+}$  catalyzes alkyl-aryl cleavage and  $\alpha$ -carbon oxidation in lignin (Tuor et al., 1992) and forms other radicals involved notably in the degradation of non-phenolic parts of lignin through the oxidation of organic sulfur compounds and unsaturated fatty acids (Kapich et al., 1999; Wariishi et al., 1989). By oxidizing phenolic compounds of lignin,  $Mn^{3+}$  also generates phenoxy-radicals, which are involved themselves in lignin depolymerization (Hofrichter, 2002). MnPs are classically described to only oxidize phenolic compounds, but they were shown to oxidize also non-phenolic lignin model compounds in the presence of additional  $Mn^{2+}$  (Chio et al., 2019). Concerning their consequences on lignin structure, MnP and laccases from *Ceriporiopsis subvermispora* have been reported to mainly cleave  $\beta$ -O-aryl ether linkages in the lignin of *Pinus taeda* (Guerra et al., 2004). MnP was also shown to trigger loss of methoxyl groups in  $^{14}C$ -labeled lignin enhancing its reactivity by increasing the phenolic content (Ander et al., 1992). In cell-free systems, MnP was shown to generate the formation of water-soluble fragments and even to mineralize 5% of  $^{14}C$ -labeled lignin (Kapich et al., 1999). MnP is the most common ligninolytic peroxidases of Basidiomycetes (Hofrichter, 2002).

Versatile peroxidases (VP) are also designated under the term hybrid peroxidases in the sense that these bifunctional enzymes present similar catalytic activities to both lignin and

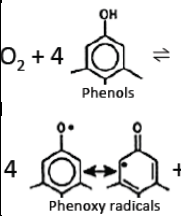
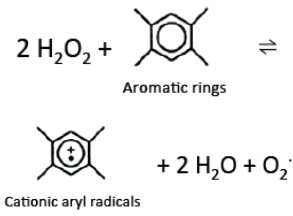
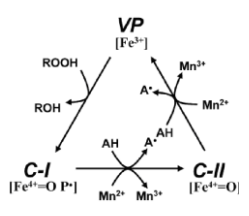
manganese-peroxidases. Indeed, the structure of VP provides multiple binding sites for different substrates enabling the oxidation of  $Mn^{2+}$  as well as both phenolic and non-phenolic aromatic compounds without a mediator. However, VP can directly degrade high reduction potential substrates without VA or oxidize independently  $Mn^{2+}$  in contrast to LiP and MnP respectively (Camarero et al., 1999). Other substrates described for VP are  $\alpha$ -keto-g-thiomethylbutyric acid (KTBA), veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols, and hydroquinones (Caramelo et al., 1999; Heinfling et al., 1998).

Dye decolorizing peroxidases (DyP) share similar catalytic properties with other previous peroxidases in the sense that they use hydrogen peroxide and mediators for substrate oxidation (Liers et al., 2014; Sugano et al., 2007). However, a DyP from *Pleurotus sapidus* was shown to be able to oxidize the substrate with the oxygen from the air and without the presence of hydrogen peroxide (Avram et al., 2018). DyP are phylogenetically distinct from other lignin-modifying peroxidases (Sugano et al., 2007) and have different sequences and structures with an  $\alpha + \beta$  ferredoxin-like fold (Singh and Eltis, 2015). More in detail, DyPs catalyze the oxidation of a variety of substrates such as phenolic and nonphenolic aromatic compounds including high redox anthraquinone dyes, lignin model compounds, and polymeric lignocellulose. However mostly DyPs demonstrate high activity against phenolic compounds but do not always accept non-phenolic compounds or more complicated lignin molecules. This catalysis occurs in the active site or on the surface of the enzyme according to the size of the substrate and on the existence of radical transfer pathways available in the enzyme (Catucci et al., 2020). According to their structures, DyPs are classified into four classes namely A, B, C, and D (Fawal et al., 2013). Unlike other lignin degrading peroxidases, DyP are produced by both bacteria (types A, B, C) and fungi (type D) (Abdel-Hamid et al., 2013).

Some studies showed synergy between different extracellular oxidoreductases in the lignin depolymerization process for instance between MnP and laccase (Galliano et al., 1991) or between MnP and LiP (Thompson et al., 1998). Thus, now that the catalytic mechanisms are better understood for each lignin-modifying enzyme, a promising research trail is at the level of the cooperation between them and with sugar-oxidizing enzymes to better understand the lignin degradation in the lignocellulosic biomass context (Hofrichter, 2002).



**Table III-9:** Main characteristics of major fungal ligninases families (phylogenetic trees were generated using the data from the fungal peroxidase database fPoxDB through the common tree tool of the taxonomy database of the NCBI website, catalytic reaction of VP reused (print) from Pérez-Boada, M., Ruiz-Dueñas, F.J., Pogni, R., Basosi, R., Choinowski, T., Martínez, M.J., Piontek, K., Martínez, A.T., 2005. Versatile Peroxidase Oxidation of High Redox Potential Aromatic Compounds: Site-directed Mutagenesis, Spectroscopic and Crystallographic Investigation of Three Long-range Electron Transfer Pathways. *Journal of Molecular Biology* 354, 385–402. <https://doi.org/10.1016/j.jmb.2005.09.047>. Copyright (2005) Elsevier)

<b>Enzyme identification</b>	Laccase (EC 1.10.3.2)	Lignin-peroxidase (EC 1.11.1.14)	Manganese-peroxidase (EC 1.11.1.13)	Versatile peroxidase (EC 1.11.1.16)	Dye decolorizing peroxidase (EC 1.11.1.19)
<b>Alternative names</b>	Urishiol oxidase	Diarylpropane oxygenase, Diarylpropane peroxidase, Ligninase I, LiP.	Mn-dependent peroxidase, MnP.	Hybrid peroxidase, Polyvalent peroxidase, VP.	DyP-type peroxidase, DyP.
<b>Catalyzed reaction</b>	 <p>Phenols</p> <p>Phenoxy radicals</p>	 <p>Aromatic rings</p> <p>Cationic aryl radicals</p>	$2 \text{Mn}^{2+} + 2 \text{H}^+ + \text{H}_2\text{O}_2 \rightleftharpoons 2 \text{Mn}^{3+} + 2 \text{H}_2\text{O}$	 <p>VP [Fe<sup>3+</sup>]</p> <p>ROOH</p> <p>ROH</p> <p>AH</p> <p>A•</p> <p>A+</p> <p>Mn<sup>3+</sup></p> <p>Mn<sup>2+</sup></p> <p>C-I [Fe<sup>4+</sup>=O P•]</p> <p>C-II [Fe<sup>4+</sup>=O]</p>	$\text{Reactive Blue 5} + 2 \text{H}_2\text{O}_2 \rightleftharpoons \text{phthalate} + 2,2'\text{-disulfonyl azobenzene} + 3\text{-}((4\text{-amino-6-chloro-1,3,5-triazin-2-yl)amino)benzenesulfonate} + 2 \text{H}_2\text{O}$

<b>Producing fungal species (reported in fPoxDB)</b>	Almost all wood decaying <b>Ascomycota</b> and <b>Basidiomycota</b> (Hoegger et al., 2006)	Fungi +- <b>Ascomycota</b>   ++Pezizomycetes    \- <i>Pyronema omphalodes</i>   \+Sordariomycetes   \- <i>Fusarium oxysporum</i> \+ <b>Basidiomycota</b> \-Polyporales ++Meruliaceae   \- <i>Phlebia brevispora</i> +-Phanerochaetaceae   +-Phanerodontia    \- <i>Phanerodontia chrysosporium</i>   +-Phlebiopsis    \- <i>Phlebiopsis gigantea</i>   \-Bjerkandera   \- <i>Bjerkandera adusta</i> \+Polyporaceae \- <i>Trametes versicolor</i>	Fungi ++ <b>Ascomycota</b>   \- <i>Pyronema omphalodes</i> \- <b>Basidiomycota</b> \-Agaricomycetes ++Trechisporales   \- <i>Sistotremastrum suecicum</i> ++Hymenochaetales   \- <i>Fomitiporia mediterranea</i> ++Corticiales   \- <i>Punctularia strigosozonata</i> ++Jaapiales   \- <i>Jaapia argillacea</i> ++Amylocorticiales   \- <i>Plicaturopsis crispa</i> ++Geastrales   \- <i>Sphaerobolus stellatus</i> +-Russulales   ++Stereaceae	Fungi +- <b>Ascomycota</b>   ++Pezizomycetes    \- <i>Pyronema omphalodes</i>   \+Sordariomycetes   \- <i>Fusarium oxysporum</i> \- <b>Basidiomycota</b> \-Agaricomycetes ++Polyporales   ++Phanerochaetaceae    \- <i>Bjerkandera adusta</i>   \-Polyporaceae   +-Dichomitus   \- <i>Dichomitus squalens</i>   +-Lentinus     \- <i>Lentinus tigrinus</i>   \-Trametes   \- <i>Trametes versicolor</i> \+Agaricales \- <i>Pleurotus ostreatus</i>	<b>Ascomycota</b> +-Lecanoromycetes   ++Teloschistales   \- <i>Gyalolechia flavorubescens</i>   \+Lecanorales   \-Cladonia   +- <i>Cladonia macilenta</i>   \- <i>Cladonia metacorallifera</i> ++Dothideomycetes   \- <i>Cenococcum geophilum</i> +-Leotiomycetes   \-Helotiales   ++Gelatinodiscaceae     \- <i>Ascocoryne sarcoides</i>   \-Sclerotiniaceae   +-Botrytis     \- <i>Botrytis cinerea</i>   \-Sclerotinia   \- <i>Sclerotinia sclerotiorum</i> ++Sordariomycetes	<b>Basidiomycota</b> +-Pucciniomycetes   \-Pucciniales   ++Melampsoraceae     \- <i>Melampsora larici-populina</i>   \+Pucciniaceae   \- <i>Puccinia graminis</i> \-Agaricomycetes ++Hymenochaetales   \- <i>Fomitiporia mediterranea</i> ++Corticiales   \- <i>Punctularia strigosozonata</i> ++Jaapiales   \- <i>Jaapia argillacea</i> +-Cantharellales   ++Botryobasidiaceae     \- <i>Botryobasidium botryosum</i>   \+Tulasnellaceae
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			<ul style="list-style-type: none"> <li>  \-<i>Stereum hirsutum</i></li> <li> \-Bondarzewiaceae</li> <li> \-Heterobasidion</li> <li>  +-<i>Heterobasidion irregulare</i></li> <li>  \-<i>Heterobasidion annosum</i></li> <li>+-Polyporales</li> <li>  ++Meruliaceae</li> <li>  \-<i>Phlebia brevispora</i></li> <li>  +-Phanerochaetaceae</li> <li>  +-Phanerodontia</li> <li>     \-<i>Phanerodontia chrysosporium</i></li> <li>  +-Phlebiopsis</li> <li>     \-<i>Phlebiopsis gigantea</i></li> <li>  \-Bjerkandera</li> <li>     \-<i>Bjerkandera adusta</i></li> <li> \-Polyporaceae</li> <li>  +-Ganoderma</li> <li> \-<i>Ganoderma sp.</i></li> <li>  +-Dichomitus</li> </ul>		<ul style="list-style-type: none"> <li> \-Sordariaceae</li> <li>  +-Sordaria</li> <li> \-<i>Sordaria macrospora</i></li> <li> \-Neurospora</li> <li>  +-<i>Neurospora tetrasperma</i></li> <li>  +-<i>Neurospora discreta</i></li> <li> \-<i>Neurospora crassa</i></li> <li>\-Eurotiomycetes</li> <li>++Verrucariales</li> <li> \-<i>Endocarpon pusillum</i></li> <li>\-Eurotiales</li> <li>\-Aspergillaceae</li> <li>+-<i>Penicillium chrysogenum</i></li> <li> \-<i>Penicillium chrysogenum</i></li> <li>\-Aspergillus</li> <li>+-<i>Aspergillus fumigatus</i></li> <li>+-<i>Aspergillus carbonarius</i></li> <li>+-<i>Aspergillus fischeri</i></li> <li>+-<i>Aspergillus terreus</i></li> </ul>	<ul style="list-style-type: none"> <li> \-<i>Tulasnella calospora</i></li> <li>++Atheliales</li> <li> \-<i>Piloderma croceum</i></li> <li>++Geastrales</li> <li> \-<i>Sphaerobolus stellatus</i></li> <li>++Sebacinales</li> <li> \-Serendipita</li> <li>  +-<i>Serendipita vermifera</i></li> <li> \-<i>Serendipita indica</i></li> <li>+-Russulales</li> <li>  ++Stereaceae</li> <li>  \-<i>Stereum hirsutum</i></li> <li> \-Bondarzewiaceae</li> <li> \-Heterobasidion</li> <li>  +-<i>Heterobasidion irregulare</i></li> <li> \-<i>Heterobasidion annosum</i></li> <li>+-Boletales</li> <li>  ++Paxillaceae</li> </ul>
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		\-Dichomitus <i>squalens</i>   +-Polyporus     +-Polyporus <i>brumalis</i>     \-Polyporus <i>arcularius</i>   +-Lentinus     \-Lentinus <i>tigrinus</i>   \-Trametes     \-Trametes <i>versicolor</i> +-Agaricales   ++Cortinariaceae     \-Hebeloma <i>cylindrosporum</i>   +-Omphalotaceae     +-Gymnopus       \-Gymnopus <i>luxurians</i>     \-Omphalotus     \-Omphalotus <i>olearius</i>   +-Strophariaceae		+-Aspergillus <i>oryzae</i> +-Aspergillus <i>flavus</i> \Aspergillus <i>clavatus</i>	\-Hydnomerulius <i>pinastri</i>   ++Sclerodermataceae     \-Scleroderma <i>citrinum</i>   ++Pisolithaceae     \-Pisolithus <i>tinctorius</i>   \-Suillaceae   \-Suillus   +-Suillus <i>brevipes</i>   \-Suillus <i>luteus</i> +-Polyporales   ++Meruliaceae     \-Phlebia <i>brevispora</i>   ++Dacryobolaceae     \-Postia <i>placenta</i>   +-Phanerochaetaceae     +-Phlebiopsis       \-Phlebiopsis <i>gigantea</i>     \-Bjerkandera     \-Bjerkandera <i>adusta</i>
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			+-Galerina       \-Galerina marginata   \Hypholoma     \-Hypholoma sublateritium  +-Tricholomataceae   \Laccaria     +-Laccaria amethystina    \-Laccaria bicolor  ++Agaricaceae   \Agaricus bisporus  \+Pleurotaceae   \-Pleurotus ostreatus \-Auriculariales ++Auriculariaceae   \-Auricularia subglabra \+Exidiaceae \ -Exidia glandulosa			\-Polyporaceae   +-Ganoderma    \-Ganoderma sp.   +-Dichomitus     \-Dichomitus squalens   +-Polyporus     +-Polyporus brumalis     \-Polyporus arcularius   +-Lentinus    \-Lentinus tigrinus   \-Trametes   \-Trametes versicolor +-Agaricales  ++Marasmiaceae     \-Moniliophthora perniciosa  ++Cortinariaceae     \-Hebeloma cylindrosporum  +-Omphalotaceae
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						+-Gymnopus       \-Gymnopus <i>luxurians</i>    \-Omphalotus     \-Omphalotus <i>olearius</i>   +-Strophariaceae   +-Galerina       \-Galerina <i>marginata</i>    \-Hypholoma     \-Hypholoma <i>sublateritium</i>   +-Amanitaceae    \-Amanita    +-Amanita <i>thiersii</i>    \-Amanita <i>muscaria</i>   +-Tricholomataceae    \-Laccaria     +-Laccaria <i>amethystina</i>    \-Laccaria <i>bicolor</i>   \+Pleurotaceae   \-Pleurotus <i>ostreatus</i>
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						<p>\-Auriculariales ++Auriculariaceae   \-Auricularia <i>subglabra</i> \+Exidiaceae \-Exidia <i>glandulosa</i></p>
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### III.3.3.2 Ligninases Phylogenetic Repartition Within the Fungal Kingdom

#### III.3.3.2.1 The Different Types of Wood Rots

Wood decaying fungi are generally divided into three main non-monophyletic groups according to the degradation mechanism involved: white-rot, brown-rot, and soft-rot fungi. The name of the different groups refers to the aspect of wood after the action of the different fungi, i.e., the majority degradation of lignin let the lighter-colored cellulose, the preferential degradation of cellulose let the brown oxidized lignin, and the action of some molds soften the wood surface (Deacon, 2013).

White-rot fungi are the most numerous wood rots and the most efficient in lignin degradation since this group is the only one able to carry out the complete mineralization to CO<sub>2</sub> (Andlar et al., 2018). This capacity relies on the fact that this group produces most of the different reported extracellular ligninases. The evolution of white rots peroxidase was even proposed to be driven by the evolution of wood lignin since the appearance of more efficient peroxidases with solvent-exposed catalytic tryptophan correlates with the diversification of angiosperms with hardwoods including dimethoxylated syringyl lignin units (Ayuso-Fernández et al., 2019). White-rot fungi include the terms white rot, corrosive rot, simultaneous rot, pocket rot, and uniform rot and are often defined as all species in the Basidiomycotina that have the capacity to degrade lignin (Blanchette, 1991). However other references also include a few species of Ascomycetes within this category (Deacon, 2013; Sigoillot et al., 2012). White-rot fungi can either selectively delignify wood, simultaneously degrade lignin and wood polysaccharides throughout the decayed wood or cause a combination of selective delignification and simultaneous decay within the same substrate (Blanchette, 1991). Indeed, *Ceriporiopsis subvermispora*, *Phellinus pini*, *Ganoderma australe*, and *Phlebia tremellosa* specifically degrade lignin and hemicellulose but not cellulose, whereas *Phanerochaete chrysosporium*, *Trametes versicolor*, *Heterobasidion annosum*, and *Irpex lacteusare* can simultaneously degrade cellulose, hemicellulose, and lignin (Weng et al., 2021).

Brown-rot (formerly red-rot) fungi represent 7% of wood-rotting Basidiomycetes and can rapidly hydrolyze the component of cellulose and hemicellulose while just partially oxidize lignin through hydroxyl radicals resulting from Fenton oxidation chemistry (Bugg et al., 2011). Indeed, these fungi produce extracellular hydroquinones that can reduce Fe<sup>3+</sup> of Fe-oxalate complex to Fe<sup>2+</sup> able to generate hydroxyl radicals by reacting with hydrogen peroxide (Jensen Jr. et al., 2001). Brown rots were shown to be able to non-selectively break the intermonomer side-chain linkages of lignin (Yelle et al., 2008) and decrease the arylglycerol-β-aryl ether linkage (Yelle et al., 2011) through this type of Fenton reactions primarily in conifer softwoods (Weng et al., 2021). Besides, brown rots have been found to generate extensive oxidative



demethylation, significant side-chain oxidation, lignin depolymerization, potentially repolymerization, limited aromatic ring cleavages, hydroxylation of aromatic rings, C $\beta$ -ether cleavage, partial side chains hydroxylation, formation of new aryl-O-aryl ether and aryl-aryl or side-chain linkages. However, in spite of all these alterations, brown-rotted lignin remains polymeric (Arantes and Goodell, 2014). Despite the different mechanisms involved in lignin degradation, DNA analyses have shown that brown rots are not separated from white rots in the Basidiomycota phylogeny (Hibbett and Donoghue, 2001). Indeed, the phylogenetic analysis of ligninolytic peroxidases showed that the mix of concerted and birth-and-death evolution processes led to the alternation of white and brown rots in the same lineages (Zhou et al., 2014). The reductive evolution of brown rots from white-rot ancestors is hypothesized to have enabled to develop a less energy-demanding wood attack strategy based on non-enzymatic processes compared to the production of complex biomass deconstructing enzymes (Arantes and Goodell, 2014).

Despite the name of the group, soft-rot fungi designate the characteristic penetration and growth of hyphae within the secondary cell walls of wood whether or not softening of the surface (Levy, 1966). Soft rots are mainly Ascomycetes decaying preferentially angiosperm hardwoods (Kuhad et al., 1997) and degrading lignin by attacking the syringyl units (Zabel and Morrell, 2020). Soft rots have been described to degrade vanillic acids and phenols, but little is known about the enzymes involved in lignin degradation and it is hypothesized that this group modifies rather than mineralize lignin (Weng et al., 2021).

#### **III.3.3.2.2 Ligninases Producers**

Laccases belong to the blue copper proteins and more precisely to the family of multicopper oxidases (MCOs) with ferroxidases, ascorbate oxidase, and ceruloplasmin. A phylogenetic comparison and classification of sequences within this family showed that laccases *sensu stricto* are produced by Basidiomycetes and Ascomycetes at least partially according to their lifestyle, whereas laccases by larger sense originate from bacteria, fungi, plants, and insects (Hoegger et al., 2006). In this study, the laccases *sensu stricto* cluster clearly according to the taxonomical association with two groups associated either to Homobasidiomycetes or to filamentous Ascomycetes. Within Homobasidiomycetes, laccases genes appeared to group according to their function notably in wood decay.

Lignin peroxidases have their genes quite limited to white-rot fungi according to the fungal peroxidase database (fPoxDB; Choi et al., 2014). Indeed only 1 gene has been assigned to each Ascomycète *Pyronema omphalodes* and *Fusarium oxysporum* over the total 43 genes

reported in fPoxDB (2021). So almost all genes documented in this bank are associated with the Polyporales order of Basidiomycetes (Table III-9).

Manganese peroxidases have drawn attention as alternative ligninolytic peroxidases to LiP due to their wider distribution among Basidiomycetes (Choi et al., 2014), although still limited to the Agaricomycetes class (Table III-9). These Basidiomycetes belong to two ecophysiological groups, namely wood-decaying fungi causing white rot and certain soil litter-decomposing fungi. Indeed, both typical wood colonizers belonging to phylogenetically older families including Meruliaceae, Coriolaceae, or Polyporaceae, and soil-litter colonizers such as Strophariaceae and Tricholomataceae produce MnP (Hofrichter, 2002). Whereas the fPoxDB lists 33 species presenting MnP genes, Hofrichter (2002) reports 56 species as producing MnP in culture according to enzyme purification or enzyme activities measurements. Thus, the number of annotated genes is likely to increase in the future.

Versatile peroxidases genes are mainly described in white-rot fungal genera *Pleurotus* and *Bjerkandera* in the literature (Hofrichter et al., 2010). However, fPoxDB reports genes in some other Basidiomycetes white rots, namely *Dichomitus squalens*, *Lentinus tigrinus*, *Trametes versicolor*, and even two Ascomycetes (Table III-9). In contrast, *Pleurotus eryngii* is not cited in fPoxDB, whereas VP was well described in this species (Camarero et al., 1999).

Dye decolorizing peroxidases are not only restricted to the fungal kingdom but are also found widely in various bacteria (Chio et al., 2019). Within fungi, the fPoxDB data show that the genes of this type of peroxidases are also widely distributed within both Ascomycetes and Basidiomycetes phyla (Table III-9). Besides, DyP structures were also characterized in the two Basidiomycetes *Termitomyces albuminosus* and *Marasmius scorodonius* (Hofrichter et al., 2010) and there is a report on DyP-like proteins in the *Auricularia auricula-judae* (Liers et al., 2010).

### **III.3.4 Conclusion and Future Perspectives**

Lignin is a highly complex, variable and high molecular weight biopolymer enclosing monomers with potential high added value applications. This structural complexity makes the characterization of lignin difficult and requires the combination of advanced techniques as shown in this chapter. The industrial processes producing technical lignins, which are among the main valorization targets, can reinforce the natural variability of lignins resulting from the random radical polymerization of monolignols in plant cell walls. Thus, the development of more adapted and effective analytical techniques appears mandatory for both the characterization of initial substrates, and the monitoring of bioconversion and biodegradation during the process. Besides physicochemical depolymerization approaches, which are effective

but with a low ecological compatibility, biological techniques try to overcome these challenges with prospect to produce bio-based synthons for green chemistry, help in bioremediation processes or improve the development of renewable energies. The fungal kingdom was widely investigated for the diversity and the particularity of its enzymatic machineries, which are considered as the most effective for lignin biodegradation as far. As a result, fungi attract attention for the development of lignin valorization processes through fungal depolymerization. However, most of the studies were carried out at bench scale as far and many challenges persist for process scale-up with yields and conversion efficiencies, which meet the industrial expectations. Envisaged ways to answer to these current limitations are: (1) the use of bioprocess engineering strategies promoting microbial diversity with complementary enzymatic activities such as aerobic granular sludge or sequencing batch biofilm bioreactor, (2) the enhancement of enzymatic catalysis through coupling with electrochemical cell, pH-responsive membranes bioreactor, graphene electron conduction, (3) improve the bioavailability and biodegradability of lignin through either biological approaches using fungal secretomes or thermochemical strategies. Fundamental studies about lignin-derived aromatics metabolism are also still needed in both bacteria and fungi to enable the development of bioprocesses involving mixed cultures and to improve microbial biodegradation efficiency as well as resistance to generated inhibitors through metabolic and genetic engineering strategies.

#### **III.4 A perspective summary of Chapter III**

A review of around 40 laboratory-scale studies on combined biological-chemical pretreatment of LCB was conducted. It showed that combined pretreatment was better than single pretreatment. However, various factors such as fungal species, culture conditions, biomass type, the severity of chemical pretreatment, and the order of subsequent pretreatments affected the overall efficiency of the process. Moreover, the correlation between biomass properties and degradability remains unclear. There were 12 studies reviewed for combined biological-chemical pretreatment of LCB for ethanol production and *Saccharomyces cerevisiae* was the predominantly used strain for fermentation. It was observed that substrate consistency and fermentation strategy also affected the biofuel yield. On the other hand, only three studies were published that used combined biological-chemical pretreatment, specifically fungal/enzyme-alkaline pretreatment, of LCB for biogas production. This showed that there is still a vast scope of research and development possible on the topic of combined pretreatment for biogas production. In the reviewed articles, most biological pretreatments were carried out using white-rot fungi due to their highly selective lignin degradation. Soft-rot fungi and endophytic fungi were only recently studied for their use in pretreatment. The usage of bacteria has not been extensively studied, even though it offers the advantage of short growth time,

because delignification was observed to be slower and limited. The other methods used for biological pretreatment are enzymes, direct addition of rumen fluid, or anaerobic fungi. But they have not been studied extensively because enzymes are expensive, and usage of strictly anaerobic microbes was limited by the difficulty in keeping the digesters strictly anaerobic. Therefore, fungal depolymerization is ongoing research for both lignin-centric biorefineries and the valorization of the products for bioenergy production. The complexity of the structure of biomass makes characterization before and after pretreatment very difficult. Various analytical techniques required to envisage the effect of the pretreatment on biomass are also reviewed and it was observed that a combination of advanced techniques are needed for better analysis. This complexity also makes developing models and simulations difficult. Since these studies have not been scaled-up nor life cycle assessments or economic assessments have not been carried out, the technology is not yet ready to be transferred to a commercial scale.

The extensive bibliography carried out helped to identify the research gap in the combined biological-chemical pretreatment of LCB for biogas production. Therefore, Fenton's reaction and fungal pretreatment were carried out individually (Chapter IV and Chapter V respectively) and combined (Chapter VI) on straw. The biomass was characterized using a combination of advanced analytical techniques and the results correlated with biogas potential to estimate the efficiency of the pretreatment method. Moreover, a bioaugmentation study helped to understand the potential of anaerobic fungi in degrading lignocellulosic biomass. This relatively new field of research provided perspectives on the potential and challenges of using anaerobic fungi which are discussed in Chapter VII.



## IV Chemical Pretreatment of Lignocellulosic Biomass

### IV.1 Optimization of oxidant concentration

#### IV.1.1 Introduction

Many studies have confirmed that the fungal action (both white-rot and brown-rot fungi) of biodegradation of lignocellulosic biomass is initiated by non-enzymatic Fenton oxidation (Arantes et al., 2012, 2011; Hammel et al., 2002; Kerem et al., 1999; Palmer and Evans, 1983). In an attempt to mimic white-rot and brown-rot fungal action, several research groups (as mentioned in Table IV-1) have used the Fenton reaction to pretreat various lignocellulosic biomass. Solution phase Fenton chemistry provides non-selective oxidation of organic compounds which could enhance cellulose bioavailability for biofuel production (Arantes et al., 2012). Typically, Fenton chemistry is based on a reaction between hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and iron ions to form reactive hydroxyl radicals ( $\bullet\text{OH}$ ) (as shown in reaction IV.1-1). The hydroxyl radical is a strong oxidant and reacts with almost all known chemical compounds. The  $\text{Fe}^{3+}$  (added or generated from  $\text{Fe}^{2+}$ ) regenerates ferrous ions and gives rise to a radical chain mechanism (as shown in reaction IV.1-2). Fenton reaction mechanism can be represented using the following reactions.



**Table IV-1:** Summary of different conditions in which Fenton pretreatment is studied

Authors	Substrate	Size of substrate	Conc. of $\text{Fe}^{2+}/\text{Fe}^{3+}$	Conc. of $\text{H}_2\text{O}_2$	Ratio of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$	pH	Temp ( $^\circ\text{C}$ )	Substrate conc (w/v)
Kato et al. (2014)	Switchgrass, Wheat Straw, Miscanthus, Corn stover	~2 mm	1.25 mM	176 mM	0.007	7	25	5%
Wang et al. (2022)	Corn stover	~2 mm	1.25 mM	176 mM	0.007	7	25	10 g in roughly 250 mL
Maamir et al. (2017)	Olive Mill Solid Waste	0.5 to 1 mm	1.5 mM	1500 mM	0.001	3	25	5%
Bhange et al. (2015)	Garden waste	1 to 5 mm	250 ppm	1000 ppm	0.250	7	50	5%
Michalska et al. (2012)	Sorghum, sida, Miscanthus	0.1–1 mm	1 g/L	25 g/L	0.040	3	25	5%
Zhang and Zhu (2016)	Sugarcane Bagasse	N.A.	20 mM	10% (w/w)	2.000	2.5	55	5%

Jung et al. (2015)	Rice straw	less than 1 mm	30 mM	2250 mM	0.013	7	25	10%
Jain and Vigneshwaran (2012)	Cotton cellulose	N.A.	0.5 mM	2%	0.250	4.2	40	0.4%
Wang et al. (2021)	Poplar	0.18 to 0.425 mm	10 mM	1000 mM	0.010	3	28	5%
Li et al. (2021)	Poplar sawdust	N.A.	0.2 mM	25 mM	0.008	4	25	6%
Yu et al. (2018)	Corn cobs	N.A.	3.3 mM	0.30 mM	0.025	2	130	10%
Chen et al. (2018)	Rice straw	(60 mesh sieve)	6 g/L	15%	-	3 to 5	25	5%
Zhang et al. (2018)	Rice straw	(150-250 $\mu$ m)	20 mM	1500 mM	0.013	7	25	10%
Jeong and Lee (2016)	Mixed hardwood	(20-80 mesh)	0.95 g/L or 27.8 g/L	29.8 g/L or 85 g/L	0.040	3	50	5%
Ämmälä et al. (2022)	Pine sawdust	0.7 mm	0.02 mg/L	2.5 g/L	0.008	3.5	N.A.	1:9
Li et al. (2016)	Corn stover	N.A.	1 g/L	30 g/L	0.033	7	20	1:10

As can be seen from Table IV-1 researchers studied the Fenton pretreatment at different pH, temperature, and reaction times according to the LCB used. It can be observed from Table IV-1, Fenton pretreatment does not require high temperature or pressure nor a high concentration of chemicals. The effectiveness of this method does not depend on the amount of ferrous salt or hydrogen peroxide but on the ratio of  $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ , pH, and reaction time. Therefore, it is easy to operate, low in cost and has the potential to be applied in lignocellulosic biorefinery. However, its poor effect on promoting enzymatic hydrolysis of the pretreated substrate makes the method inefficient to be done individually. Therefore, Fenton pretreatment is studied in combination with other methods such as dilute alkali, dilute acid, ultrasound, and hydrothermal so far (Bhange et al., 2015; Jung et al., 2015; Zhimin Wang et al., 2022). Some of these methods have the disadvantage of harsh conditions and therefore, the Fenton pretreatment was conducted in combination with moderate conditions of physical pretreatment and autoclaving and discussed in this chapter. Of the reviewed articles in Table IV-1, Kato et al. (2014) studied Fenton pretreatment on wheat straw and found a 2.33-fold increase in fermentation gas production. Therefore, in this study, based on the  $\text{Fe}^{2+}/\text{H}_2\text{O}_2$  ratio used by Kato et al. (2014), the optimum range of Fenton reagent concentration was studied for straw for better lignin degradability.

## IV.1.2 Materials and Methods

### IV.1.2.1 Materials

All chemicals of reagent grade were obtained from VWR, Rosny-sous-Bois Cedex, France, and used without further purification. Demineralized water (18 M $\Omega$ ·cm) was used to make all the solutions. Straw samples were obtained from Hamiform (in early 2019), a commercial brand that sells it as bedding for rodents and it mainly consists of wheat straw mixed with other straw.

### IV.1.2.2 Physical Pretreatment

Straw was ground using a laboratory blender (Waring commercial) and sieved through a 2 mm mesh-size sieve and then again through a 630  $\mu$ m sieve to recover the retentate.

### IV.1.2.3 Autoclaving Process

The biomass was then added to Erlenmeyer flasks and soaked in demineralized water to obtain a 5% (w/v) concentration and was autoclaved (Vertical Autoclave, LEQUEUX, Paris, France) at 121°C and 2 bars for 20 min.

### IV.1.2.4 Fenton Pretreatment

Three different concentrations of Fenton reagents, namely low, medium, and high concentrations were studied with the same Fe<sup>2+</sup> to H<sub>2</sub>O<sub>2</sub> ratio of 0.007 as shown in Table IV-2. Fenton pretreatment study was conducted in demineralized water (DW) at pH 7 with 5% (w/v) substrate consistency i.e., 5 g of straw in 100 mL DW. The flasks were then left to shake on an orbital shaker at room temperature for 24 h. The liquid phase was then removed by vacuum filtration and harvested straw was washed with demineralized water and dried at 40°C for 2–3 days. Control experiments were conducted in the same conditions without any FeCl<sub>2</sub> or H<sub>2</sub>O<sub>2</sub>. All experiments were conducted in triplicates.

**Table IV-2:** Concentration of Fenton reagents used for the optimization study

Control	Low concentration	Medium concentration	High concentration
No oxidants	1.065 mM Fe <sup>2+</sup> (63.55 mg) + 0.15 M H <sub>2</sub> O <sub>2</sub> (5.1 mL)	2.09 mM Fe <sup>2+</sup> (127.10 mg) + 0.30 M H <sub>2</sub> O <sub>2</sub> (10.2 mL)	4.19 mM Fe <sup>2+</sup> (254.21 mg) + 0.59 M H <sub>2</sub> O <sub>2</sub> (20.4 mL)



### **IV.1.2.5 Characterization methods**

#### **IV.1.2.5.1 Total solids and Elemental Composition**

The total solids (TS) content of the straw fractions and the inoculum was determined after drying the biomass at 105°C for 20 h. The tests were done in triplicates.

Carbon (C), hydrogen (H), nitrogen (N), sulfur (S), and oxygen (O) contents were obtained using Flash 2000 FlashSmart Elemental Analyzer (Thermo Fisher Scientific, Courtaboeuf Cedex, France). Approximately 1.0 mg samples each were added to tin containers along with a few mg of Vanadium for C, H, N, and S analysis and silver containers for oxygen analysis. All tests were conducted in triplicates.

#### **IV.1.2.5.2 Lignin Composition Analysis**

The lignin compositional analysis was conducted using the Laboratory Analytical Procedure (LAP) published by the National Renewable Energy Laboratory (NREL) which is a two-step acid hydrolysis method (Sluiter et al., 2012). In short, 300 ± 10 mg of untreated or pretreated straw was hydrolyzed with 3 mL of 72% (w/w) H<sub>2</sub>SO<sub>4</sub> at 30°C for 1 h, followed by dilution with demineralized water to 4% (w/w) H<sub>2</sub>SO<sub>4</sub> and autoclaved at 121°C for 1 h. The hydrolysate was then vacuum filtered and the liquid fraction was used for determining the acid-soluble fraction of lignin (ASL) using a UV spectrophotometer at 278 nm (Absorptivity = 30 L/g cm). The solid fraction was washed with demineralized water and dried at 105°C for 15 h and then transferred to a furnace and the temperature was ramped to 575°C for 3 h. By measuring the weight before and after the ashing in the furnace (Nabertherm), acid-insoluble lignin (AIL) was calculated. The % total lignin content of a sample is calculated as the sum of ASL and AIL. The tests were done in triplicates.

#### **IV.1.2.5.3 Attenuated Total Reflectance (ATR)**

The presence of various functional groups was assayed using a Nicolet iS5-iD3 ATR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For this, analysis was performed over the spectral range of 4000–400 cm<sup>-1</sup> with 16 scans. The analysis was carried out in triplicates and averaged. A baseline correction was then performed in LabSpec software with the parameters, type: linear; Degree: 3; Points: 10 and 0-1 normalization in Origin 2018 software.

#### **IV.1.2.5.4 Scanning Electron Microscopy (SEM)**

The surface morphologies of raw and pretreated straw were characterized by SEM using Quanta 250 FEG system (FEI Company, Hillsboro, OR, USA). Before SEM observation, a thin

layer of 5 nm thickness of platinum was coated on the samples to have higher resolution and magnification and to avoid beam damage to sensitive samples.

### IV.1.3 Results and Discussion

#### IV.1.3.1 Elemental composition

The elemental composition of the straw in control and pretreated samples does not vary. The values obtained (as shown in Table IV-3) are similar to that found in the literature such as Demirbaş (1997), Singh et al. (2014), and Yang and Wu (2009). The elemental contents especially carbon and hydrogen contents partially determine the energetic properties of agro-residues (Demirbaş, 1997). As observed in the analysis, the straw biomass contains a higher proportion of C and H contents, which increases the energy value (i.e., calorific value). The use of straw for biogas production is restricted by its high C/N ratio. However, co-digestion with high nitrogen substrates like cow manure or food waste will provide additional improvement in biogas yield (Rahmani et al., 2022).

**Table IV-3:** Total solids and Elemental composition of straw pretreated with different oxidant concentrations

Sample	%TS	% C (%TS)	% H (%TS)	% N (%TS)	% S (%TS)	% O (%TS)
Control	98.8 ± 0.4	45.9 ± 0.2	5.8 ± 0.0	0.4 ± 0.1	0.1 ± 0.2	40.7 ± 1.2
Low conc.	97.7 ± 0.8	45.3 ± 0.2	5.7 ± 0.2	0.3 ± 0.0	0.0 ± 0.0	43.7 ± 0.4
Medium conc.	98.3 ± 1.0	46.1 ± 0.2	5.8 ± 0.1	0.4 ± 0.0	0.0 ± 0.0	43.0 ± 0.2
High conc.	98.5 ± 0.5	45.5 ± 0.4	5.8 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	43.2 ± 0.2

#### IV.1.3.2 Lignin composition

From Figure IV-1, it can be observed that there was an overall reduction in lignin content as compared to control in all conditions studied. An average ASL degradation of 13.2 ± 2.4 % and AIL degradation of 22.9 ± 2.0 % was observed across all three conditions as compared to the control. Kato et al. (2014) observed 8.76% and 16.8% degradation of AIL and ASL respectively after Fenton pretreatment of wheat straw. The lignin degradation (Figure IV-1) is observed to be slightly higher when pretreated with low (20% lignin degradation) and medium concentration (22% lignin degradation) of Fenton reagents as compared to the higher concentration (19% lignin degradation) studied. Therefore, even the lowest concentration of

oxidants is enough to obtain similar lignin degradation. This would reduce the amount of oxidants required when the pretreatment process needs to be scaled up.

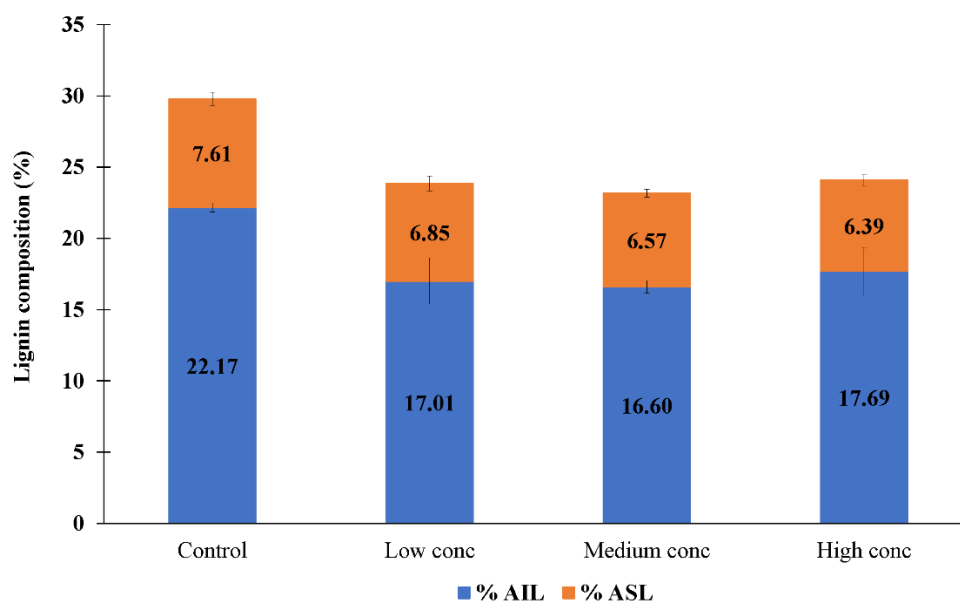


Figure IV-1: Lignin composition in terms of %ASL and %AIL of the straw pretreated in different conditions

#### IV.1.3.3 Attenuated total reflection spectroscopy

To investigate the chemical modifications due to pretreatment, an ATR analysis of the samples was carried out. The spectra obtained are shown in Figure IV-2 and each peak is associated with the specific functional groups as shown in Table III-7. Several peak ratios from ATR-FTIR have been used to highlight the changes in the cellulose structure due to pretreatment. The lateral order index (LOI) gives the empirical crystallinity index and is defined as the ratio between the amorphous region in cellulose ( $897\text{ cm}^{-1}$ ) and the crystalline structure of cellulose ( $1420 - 1430\text{ cm}^{-1}$ ). Lower values of LOI mean lower crystallinity and higher digestibility. Hydrogen bond intensity (HBI) is determined as the ratio of intensities at O-H stretching, H-bonds between molecules at  $3400\text{ cm}^{-1}$ , and CH rocking vibration of the glucose ring at  $1320\text{ cm}^{-1}$ . HBI provides information about crystallinity and the amount of bound water (the degree of intermolecular regularity). Cross-linked lignin (CLL), on the other hand, provides information on the proportion of lignin with condensed and cross-linked structures which is a characteristic feature of the concentration of guaiacyl. It is calculated as the ratio between band intensities at  $1600$  and  $1508\text{ cm}^{-1}$  (Auxenfans et al., 2017). The higher the CLL value, the higher the condensed and cross-linked structures present in the biomass. The extent of hydrolysis of lignocellulosic biomass can also be estimated based on the lignin-to-cellulose ratio (L/C). It is obtained by the ratio of absorption intensities at  $1510$  and  $898\text{ cm}^{-1}$ . The low lignin to cellulose

(L/C) ratio implies a higher degree of digestibility (Auxenfans et al., 2017; Gaur et al., 2015; Scharer and Moo-Young, 1979).

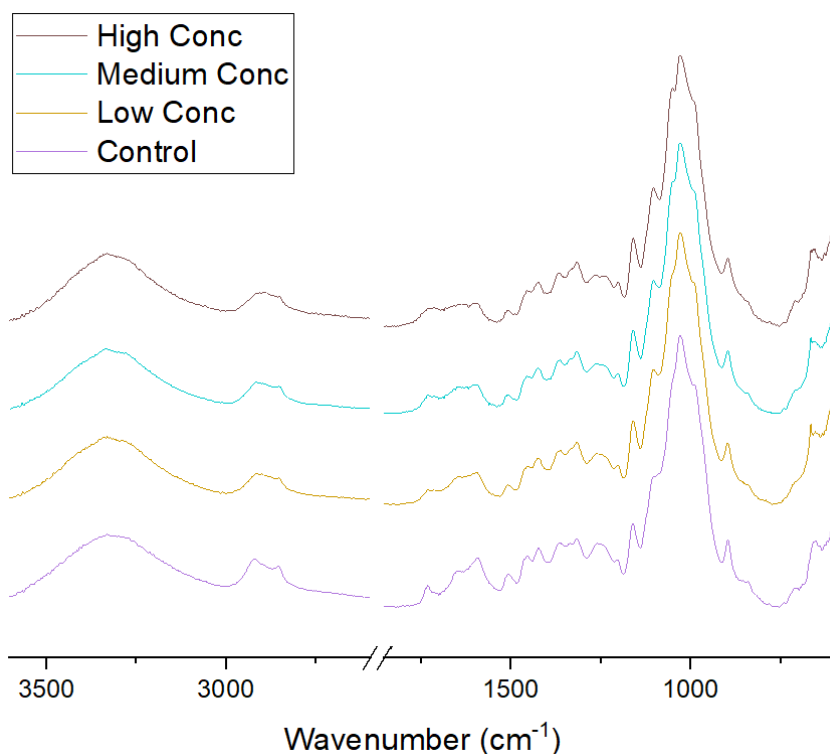


Figure IV-2: ATR spectra of control and pretreated straw with increasing pretreatment severities

Figure IV-3 provides the ratio of LOI, HBI, CLL, and L/C. The LOI values are observed to decrease upon pretreatment. With the increasing severity of the pretreatment, the LOI decreased further, showing the cellulose structure was affected to a different extent. Whereas, the HBI ratio did not vary much with the control and pretreated sample. On the other hand, the CLL ratio reduced from 0.67 in the control to 0.61 in the low-concentration oxidant pretreated sample. Gaur et al. (2015) saw a decrease in CLL ratio from 1.18 to 1.09 in pretreated corn stover and it reflected an increase in glucose release from 0.33 to 0.69 (g/g glucan) upon enzymatic saccharification. However, the L/C ratio also decreased with increasing severity of pretreatment (Figure IV-3), showing the higher degree of digestibility of the pretreated samples as compared to the control. Alexandropoulou et al. (2017) observed a similar decrease in the L/C ratio from 0.84 to 0.76 as the fungal pretreatment increased from 5 to 30 days.

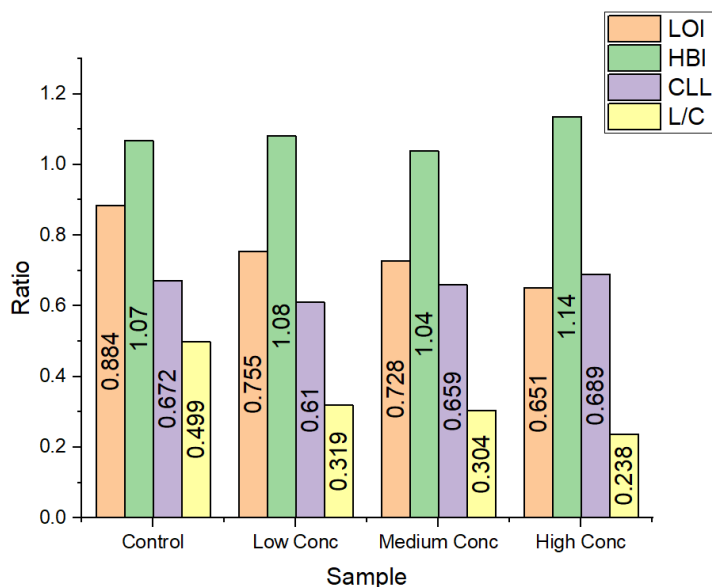


Figure IV-3: Cellulose and lignin related properties of the control and pretreated straw

#### IV.1.3.4 Scanning Electron Microscopy

The surface morphology of straw before and after Fenton pretreatment was visually examined using SEM analysis. As seen in Figure IV-4 (A1, A2), the control has a slightly damaged surface because of the autoclaving process. After Fenton pretreatment at low concentration (Figure IV-4 – B1, B2), pores are very visible which are created by the dissolution of lignin and hemicellulose content. Even at the low concentration of oxidants, we can confirm that radical species were generated that were able to modify and degrade biomass. The porous structure provides increased surface area for the microbial attack that promotes further degradation compared to the untreated substrate (Katukuri et al., 2017; Olli Joutsimo, 2004). Lesser number of pores are visible in the straw pretreated at a medium concentration of oxidant pretreatment (Figure IV-4 – C1, C2) whereas the modifications to structural changes are very minimal in straw pretreated at a high concentration (Figure IV-4 –D1, D2). This could be because there were side reactions that occurred at high concentrations of oxidant pretreatment which prevented the generated radical species to degrade biomass. Overdosage of  $\text{Fe}^{2+}$  ions beyond the catalytic amounts is shown to lead to radical self-scavenging by  $\text{Fe}^{2+}$  and cause a decrease in the degradation rates. Moreover, the rapid and large addition of  $\text{H}_2\text{O}_2$  could increase the probability of side reactions. This probability of side reactions could have caused the color change in higher concentration study as compared to low concentration (Figure IV-5). Whereas, in the case of lower oxidant concentration, there is gradual consumption of  $\text{H}_2\text{O}_2$  over an extended time (Bhange et al., 2015).

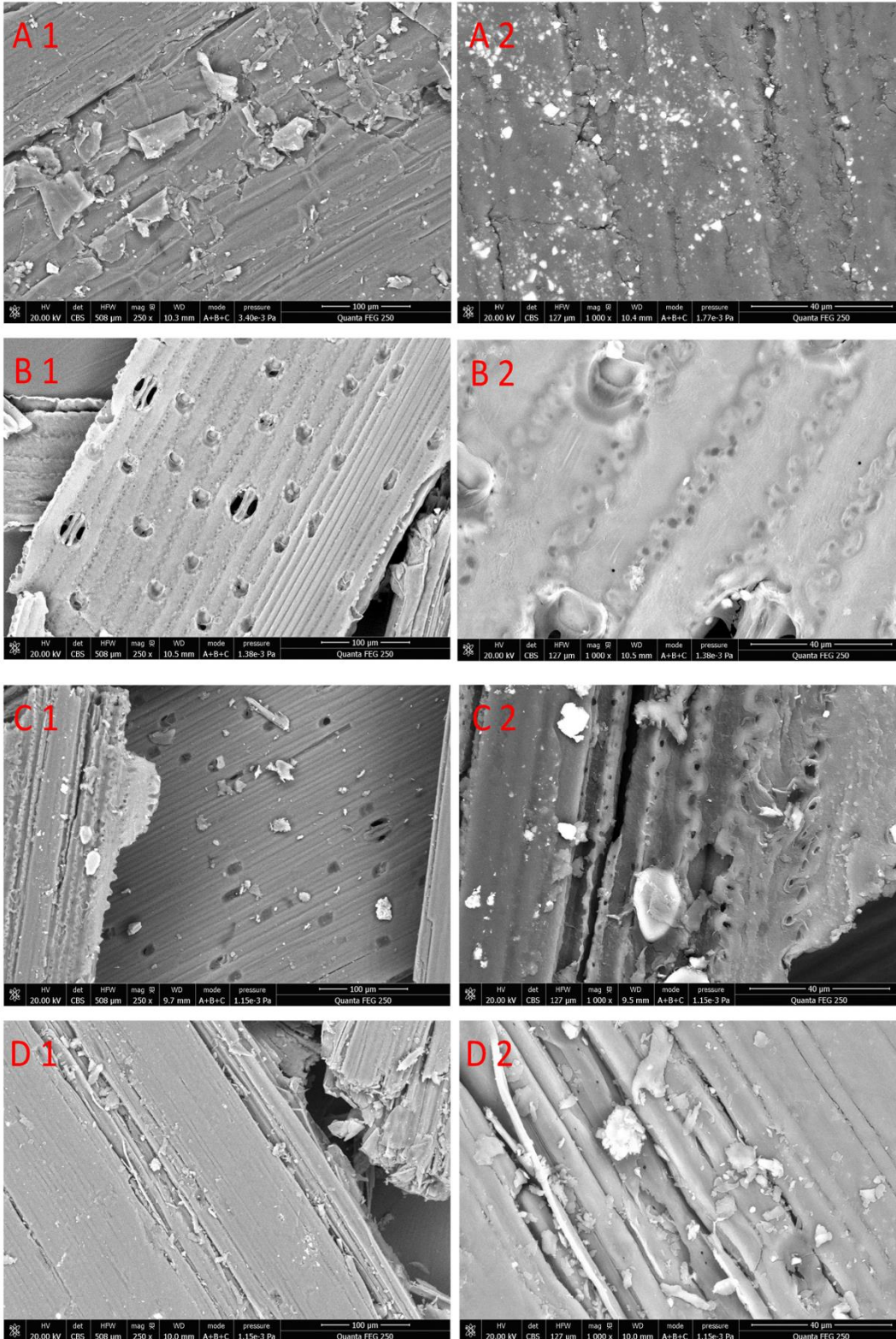


Figure IV-4: SEM images of control straw (A), straw pretreated at low concentration (B), medium concentration (C), and high concentration (D). The images are taken at 250x magnification (1) and 1000x magnification (2)

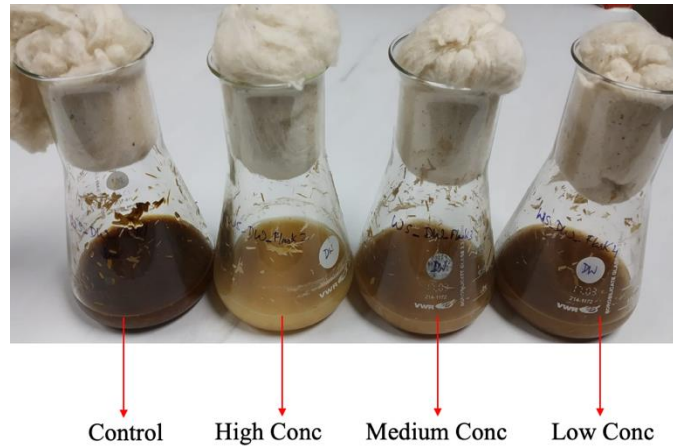


Figure IV-5: Image of the oxidant pretreatment set up after incubation at room temperature for 24 hours in an orbital shaker

Therefore, further experiments were conducted with the lowest concentration studied as it would help to reduce the quantity of chemicals used when the process is scaled up without any side reactions.

#### IV.1.4 Conclusion and Perspectives

A Fenton-based process with three different concentrations was used to treat straw. The results showed that a low concentration of oxidants was enough to cause structural changes in the straw to improve its digestibility as compared to the control. However, according to some literature, a pH range of 3 – 4.5 is better suitable for the Fenton reaction. Therefore, further studies on the Fenton pretreatment were carried out at pH 3. Nevertheless, the influence of size reduction and autoclaving on the structural changes will alter the effect of Fenton pretreatment on the biomass. This is further studied in the next part of this chapter (IV.2) where the contribution of size reduction and autoclaving process to the structural changes in straw helped to estimate the actual influence of Fenton pretreatment on biomethane production.

## IV.2 Chemically and physically pretreated straw in moderate conditions: Poor correlation between biogas production and commonly used biomass characterization

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Article

### Chemically and Physically Pretreated Straw in Moderate Conditions: Poor Correlation between Biogas Production and Commonly Used Biomass Characterization

Shruthi Meenakshisundaram <sup>1</sup>, Vincenzo Calcagno <sup>1,2</sup>, Claire Ceballos <sup>1</sup>, Antoine Fayeulle <sup>1</sup>, Estelle Léonard <sup>1</sup>, Virginie Herledan <sup>2</sup>, Jean-Marc Krafft <sup>2</sup>, Yannick Millot <sup>2</sup>, Xiaojun Liu <sup>1</sup>, Claude Jolivalt <sup>2,\*</sup> and André Paus <sup>1,\*</sup>

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<sup>1</sup> Université de Technologie de Compiègne, ESCOM, TIMR (Integrated Transformations of Renewable Matter), Centre de Recherche Royallieu—CS 60 319, F-60203 Compiègne CEDEX, France

<sup>2</sup> Sorbonne Université, CNRS, Laboratoire de Réactivité de Surface (LRS), 4 place Jussieu, F-75005 Paris, France

\* Correspondence: [claude.jolivalt@sorbonne-universite.fr](mailto:claude.jolivalt@sorbonne-universite.fr) (C.J.); [andre.paus@utc.fr](mailto:andre.paus@utc.fr) (A.P.); Tel.: +33-(0)1-44-27-60-13 (C.J.); +33-(0)3-44-23-44-57 (A.P.)

**Abstract:** Straw is a substantial agricultural by-product for biogas production. Hydrolysis of straw is found to be a rate-limiting step during its anaerobic digestion and could be enhanced by pretreatment. In this paper, the effect of various combinations of particle size reduction, autoclaving, and low-level Fenton reaction was studied on straw for biogas production. Grinding of straw contributed to the maximum increase in the biomethane potential. Only Fenton or only the autoclave process improves the kinetics slightly but does not considerably improve the biomethane potential. Combining autoclaving and low-concentration Fenton pretreatment considerably improves the BMP values. Lignin content, CHNSO elemental analysis, Scanning Electronic Microscopy (SEM), Simon's staining, infrared spectroscopy (DRIFT and ATR), Nuclear magnetic resonance spectroscopy, and wide-angle X-ray diffraction analysis (WAXD) were used to characterize the physical and chemical changes of straw due to pretreatment. Results show a poor correlation between biogas production and the different physical and chemical biomass characteristics. It makes it difficult to explain the outcome of various pretreatment methods applied to biomass. Without further improvement and development of analytical techniques, the prediction of the biomethane potential of a feedstock with the aid of pretreatment can only be considered in case-by-case studies.

**Keywords:** anaerobic digestion; biomass; straw pretreatment; size reduction; autoclave; Fenton reaction

#### IV.2.1 Introduction

Straw is a substantial agricultural by-product around the world, making it an attractive substrate for biogas production via anaerobic digestion (AD). The lignocellulosic biomass (LCB) such as straw contains three main polymers called cellulose, hemicellulose, and lignin which are intertwined making it recalcitrant and hindering sugar release (Sun et al., 2013). Due to this complex structure, straw has low degradability which results in low biogas yield. The



biomass undergoes four steps in the anaerobic digestion process to produce methane. First, hydrolysis occurs where hydrolytic bacteria in the inoculum help to depolymerize carbohydrates (hemicellulose and cellulose), proteins, and lipids into monomers. Second, the fermentative bacteria convert simple sugars into volatile fatty acids (VFA) such as propionic acid and acetic acid, and this step is called acidogenesis. Then, acetogenesis takes place in which the acetogenic bacteria convert the VFA into hydrogen, carbon dioxide, and acetate. Finally, the methanogenic archaea convert acetate (acetoclastic methanogenesis) or hydrogen plus carbon dioxide (hydrogenotrophic methanogenesis) into methane and carbon dioxide (Adney et al., 1991; Zhongzhong Wang et al., 2022).

Hydrolysis is found to be a rate-limiting step in the conversion process. Nevertheless, a pretreatment step can help to make the organic matter in the feedstock more accessible to microbial attack which increases its biodegradability (Adney et al., 1991; Y. Li et al., 2019; Sun et al., 2013). Physical, chemical, physicochemical, and biological pretreatments are generally used alone or in combination to enhance biofuel yield (Baruah et al., 2018). Generally, physical pretreatment includes mechanical, thermal, and irradiation techniques. Mechanical pretreatment such as grinding helps to reduce the size of biomass which cannot only increase the surface area for microbial degradation but also be useful to intensify the AD process. Size reduction could avoid the problems of floatation and strengthen the mixing, heat, and mass transfer in the downstream process (Coarita Fernandez et al., 2020; Kaur, 2022). It consequently boosts the digester feeding conditions and reduces the digester size. Thermal pretreatment is carried out by heating the biomass at a certain temperature (50–240°C) and pressure for enhancing particulate organic matter. Autoclaving is a thermal pretreatment that operates at a temperature of 121°C and an absolute pressure of 2 bar (Feng et al., 2021). When the temperature is elevated to 160–240°C, it is known as liquid hot water pretreatment. When using moderate temperatures (50–100°C), the thermal pretreatment is carried out for several hours to days. Even though liquid hot water pretreatment is known to improve hemicellulose hydrolysis, it can result in an inhibitor formation whereas, autoclaving does not produce inhibitors (Antczak et al., 2022; Deepanraj et al., 2017). The energy input of physical pretreatment methods should be taken into account which reduces the energy gain from biofuel yield surplus induced by that pretreatment. Chemical pretreatment is further classified as dilute acid, alkaline, oxidative pretreatment, and organic solvent pretreatment. Based on the chemical used and operating parameters, the mode of action of LCB pretreatment varies, primarily removing lignin or hemicellulose (Abraham et al., 2020). Alkaline pretreatment is considered an effective and low-cost method to improve the biogas performance of lignocellulosic feedstock. NaOH, KOH, and Ca(OH)<sub>2</sub> are commonly used chemicals for alkaline pretreatment. Nonetheless, the concern of

sodium discharge in the process effluent and digestate limits the NaOH use. On the other hand, KOH (strong base)-treated anaerobic digestate can be used as fertilizers but the high chemical loading and toxicity to microbes are also a concern. Although  $\text{Ca}(\text{OH})_2$  is low-cost and safe to use, the weak alkali alone cannot improve biomass digestion significantly. Due to the inherent demerits of conventional pretreatments as described previously and in Anukam and Berghel (2021), combining two or more pretreatment techniques will help to conduct the individual steps at mild conditions while reducing the severity of the disadvantages (Kumar and Sharma, 2017; Meenakshisundaram et al., 2021). Uellendahl et al. (2008) studied wet oxidation pretreatment for corn, miscanthus, and willow and concluded that the pretreatment was efficient for improving the biogas yield of only miscanthus and willow and not corn. This is because the relative abundance of the three polymers in LCB varies depending on the type, species, and even source of biomass (Bajpai, 2016). Each pretreatment method specifically affects the different components of the biomass and the efficiency can vary even if the same pretreatment step is applied to the same biomass from different sources, thereby making it quite difficult to compare (Meenakshisundaram et al., 2021). One has to note that it is still not clear why some pretreatment methods are efficient for enhancing biogas production while others are not.

Studying the properties of biomass using characterization techniques could help to determine the feasibility and viability of its products. However, the characterization of biomass is also challenging as each technique has its advantages and disadvantages. Thus, the limitations of one technique are compensated by the merits of other techniques. Consequently, a wide variety of state-of-the-art analytical techniques are required to interpret the efficiency of the pretreatment. The analytical methods need to be chosen based on studying the biomass at different levels—functional groups, structural changes, surface morphology, and fiber elemental content to achieve a full understanding of the physical and chemical underpinnings of biomass as it undergoes bioconversion (Anukam and Berghel, 2021). Lignin compositional analyses are generally carried out using standardized protocols provided by NREL (Sluiter et al., 2012), Van Soest (P.J. Van Soest and Wine, 1968), or TAPPI (Technical Committee ISO/TC, 2020) method. Spectroscopic methods such as Fourier-transform infrared spectroscopy (FT-IR)/attenuated total reflectance (ATR), Raman, and nuclear magnetic resonance spectroscopy (NMR) are used to determine variation occurring in functional groups present. To analyze the morphological changes related to enzyme accessibility, scanning electron microscope (SEM), transmission electron microscope (TEM), and atomic force microscope (AFM) are used. X-ray diffraction (XRD) and solid-state NMR techniques provide data on the physical properties of biomass such as crystallinity, crystallite size, and fiber diameter. Size exclusion chromatography (SEC) is used to examine the molecular distribution

and polydispersity ratio. Gas chromatography-mass spectrometry (GC-MS) helps to analyze the S/G unit ratio and phenolic content of lignin (Meenakshisundaram et al., 2022). The heterogeneity of the biomass calls for diligent sample preparation and careful analysis to have precise analytical results (Dayton and Foust, 2020). Based on these analyses, various researchers have concluded different factors or components that influence the methane yield in their study. This contradictory nature of results from various studies makes it difficult to define which characteristics are important for an efficient pretreatment and enhancement of biofuel yield.

Since physical and chemical pretreatments are the widespread strategies to improve biomass digestibility, this paper aims to study their combination in improving the biochemical methane potential (BMP) of straw. Size reduction is unavoidable for preparing the feedstock for biorefinery processes and the most common technique used is grinding (Oyedemi et al., 2020). Therefore, the improvement in the performance of AD due to small changes in the extent of size reduction by grinding is assessed. Autoclaving is typically done before biological or enzyme pretreatment to maintain sterility and therefore, its effect on biogas production is also assessed (Liu et al., 2017). Of the chemical strategies, Fenton pretreatment is interesting as it mimics the natural process used by fungi to decay lignocellulosic material (Jung et al., 2015). Fenton reaction occurs between hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and ferrous ion ( $\text{Fe}^{2+}$ ), which produces a hydroxyl ( $\cdot\text{OH}$ ) radical capable of oxidizing organic compounds. This degradation of organic compounds subsequently produces more hydroxyl radicals and thereby initiates a chain reaction (Bhange et al., 2015). Therefore, the objectives of the present study are (1) to apply combined physical and chemical pretreatment methods on straw (2) to realize biochemical methane potential tests (BMP tests) to seek the relevant biogas enhancement, (3) to perform the characterization of the biomass pretreated or not and (4) to look into the correlation between the biogas production and the biomass characteristics.

## **IV.2.2 Materials and Methods**

### **IV.2.2.1 Materials**

All chemicals of reagent grade were obtained and used without further purification, except for Direct Orange (DO, Pontamine Fast Orange 6RN) which was purified according to the procedure described in Section IV.2.2.7.2. Ferrous chloride tetrahydrate ( $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 30%), and sulfuric acid ( $\text{H}_2\text{SO}_4$ , 96%) were purchased from VWR, Rosny-sous-Bois Cedex, France. Direct Blue (DB, Pontamine Fast Sky Blue 6BX) and DO, sodium phosphate dibasic heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\geq 98\%$ ), sodium phosphate monobasic monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ,  $\geq 98\%$ ), sodium chloride ( $\text{NaCl}$ ,  $\geq 99\%$ ), hydrochloric

acid (HCl, 37%), and sodium hydroxide (NaOH,  $\geq 98\%$ ) were obtained from Sigma Aldrich, Saint-Quentin-Fallavier Cedex, France. 100 K ultrafiltration membranes were obtained from Amicon (Amicon Inc., Beverly, MA, USA). Diamond powder average size of 0.6 mm was purchased from Presi (Presi, Eybens, France). Distilled water (18 M $\Omega$ ·cm) was used to make all the solutions.

Straw samples were obtained from Hamiform (in December 2021), a commercial brand that sells it as bedding for rodents and it mainly consists of wheat straw mixed with other straw.

#### **IV.2.2.2 Physical Pretreatment**

Straw was ground using a laboratory blender (Waring commercial) and sieved into two-size fractions. The smaller size fraction (denoted as S) consisted of particles obtained by sieving through a 2 mm mesh-size sieve and then again through 630  $\mu\text{m}$  to recover the retentate. While the larger size fraction (denoted as L) consisted of straw particles obtained after sieving first through 4 mm and then through 2.5 mm sieve to recover the retentate.

#### **IV.2.2.3 Autoclaving Process**

The two size fractions of biomass were added to different Erlenmeyer flasks and soaked in distilled water to obtain a 5% (w/v) concentration. Half of the total number of flasks of each size fraction were autoclaved (denoted as A) (Vertical Autoclave, LEQUEUX, Paris, France) at 121°C and 2 bars for 20 min while others were not (denoted as NA).

#### **IV.2.2.4 Fenton Pretreatment**

The pH of the suspension of straw in distilled water was then reduced to 3 with dilute sulfuric acid (1 M). 190.65 mg of FeCl<sub>2</sub> and 15.3 mL of H<sub>2</sub>O<sub>2</sub> were added to each flask containing 15 g straw and 300 mL of distilled water at pH 3 (denoted as F). The ratio of Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> was chosen based on Kato et al. (2014). The flasks were then left to shake on an orbital shaker at room temperature for 24 h. The liquid phase was then removed by vacuum filtration and harvested straw was washed with distilled water and dried at 40°C for 2–3 days. Control (denoted as C) experiments were conducted in the same conditions without any FeCl<sub>2</sub> or H<sub>2</sub>O<sub>2</sub>. All experiments were conducted in triplicates.

#### **IV.2.2.5 Chemical and Physio-Chemical Characterization**

##### **IV.2.2.5.1 Determination of the Total Solid and Volatile Contents of Samples**

Total solids (TS) and volatile solids (VS) content of the straw fractions and the inoculum were determined after drying the biomass at 105°C for 20 h and 550°C for 2 h, respectively. The tests were done in triplicates.

#### **IV.2.2.5.2 CHNSO Elemental Composition**

Carbon (C), hydrogen (H), nitrogen (N), sulfur (S), and oxygen (O) contents were obtained using Flash 2000 FlashSmart Elemental Analyzer (Thermo Fisher Scientific, Courtaboeuf Cedex, France). Approximately 1.0 mg samples each were added to tin containers along with a few mg of Vanadium for C, H, N, and S analysis and silver containers for oxygen analysis. All tests were conducted in triplicates.

#### **IV.2.2.5.3 Lignin Composition Analysis**

The lignin compositional analysis was conducted using the Laboratory Analytical Procedure (LAP) published by the National Renewable Energy Laboratory (NREL) which is a two-step acid hydrolysis method (Sluiter et al., 2012). In short,  $300 \pm 10$  mg of untreated or pretreated straw was hydrolyzed with 3 mL of 72% (w/w)  $\text{H}_2\text{SO}_4$  at  $30^\circ\text{C}$  for 1 h, followed by dilution with distilled water to 4% (w/w)  $\text{H}_2\text{SO}_4$  and autoclaved at  $121^\circ\text{C}$  for 1 h. The hydrolysate was then vacuum filtered and the liquid fraction was used for determining the acid-soluble fraction of lignin (ASL) using a UV spectrophotometer at 278 nm (Absorptivity = 30 L/g cm). The solid fraction was washed with distilled water and dried at  $105^\circ\text{C}$  for 15 h and then transferred to a furnace and the temperature was ramped to  $575^\circ\text{C}$  for 3 h. By measuring the weight before and after the ashing in the furnace (Nabertherm), acid-insoluble lignin (AIL) was calculated. The % total lignin content of a sample is calculated as the sum of ASL and AIL. The tests were done in triplicates.

#### **IV.2.2.6 Biochemical Methane Potential (BMP) Test**

##### **IV.2.2.6.1 Realization of BMP Tests**

The BMP tests were performed using an automatic methane potential test system (AMPTS II, Automatic Methane Potential Test System, Bioprocess Control AB, Lund, Sweden). The AMPTS II is a standardized analytical device designed for the online measurement of biomethane obtained from the anaerobic digestion of biodegradable substrates. It consists of 15 parallel reactors of 500 mL each that are connected to separate gas flow meters through an 80 mL trap bottle of 3 M sodium hydroxide solution used for absorbing  $\text{CO}_2$  from the biogas. The remaining gas after scrubbing is measured using the gas flow counters which are connected to the data analysis and acquisition system. The experiments were conducted with 400 g of the total reaction medium, in which substrates and inoculum were mixed at a weight ratio of 1:3 in terms of volatile solids (VS) at mesophilic conditions ( $37^\circ\text{C}$ ) with continuous mixing. All tests were performed in triplicate. Total solids (TS) and VS were determined for both substrates and the inoculum as described in Section IV.2.2.5.1. Blank samples consisting of only inoculum were also carried in triplicates. The net methane

production of the substrate was determined by subtracting the methane production of the blank (inoculum) from the substrate sample (substrate + inoculum). BMP tests were run for 57 days and the cumulative methane production was recorded. The quality of BMP tests was assured by a positive control in triplicate (cellulose + inoculum) as suggested by Holliger et al. (2016).

The inoculum was obtained from a large-scale anaerobic digester digesting agricultural residues located in Coudon (Oise), France. To remove large and undigested particles in the inoculum, it was filtered through a 1 mm porosity sieve and then left to stabilize for a week at room temperature. FOS/TAC, the ratio of volatile fatty acids (Fluchtige Organische Sauren in German) to total alkalinity (Total Anorganic Carbon in German) (Nkuna et al., 2021), was determined using the Titralab AT1000 series (HACH) instrument. The inoculum had a FOS/TAC ratio of  $0.13 \pm 0.02$  and the percentage of TS and VS were  $7.5 \pm 0.2$  and  $65.2 \pm 0.5$ .

#### **IV.2.2.6.2 Statistical Analysis and Kinetics of Biomethane Production**

Statistical significance in the difference between the net biomethane production between all the samples was studied using ANOVA (single factor test) at 0.05 level in XLSTAT (Addinsoft, Paris, France), an add-in software on Microsoft Excel.

To better understand the influence of the pretreatment methods used on the anaerobic digestion efficiency, a first-order kinetic model (Equation IV.2-1) was used as the degradation of biomass is assumed to follow a first-order decay.

$$V(t) = V_{\max} (1 - \exp^{-kt}) \quad (\text{Eq.IV.2-1})$$

where  $t$  [days] is the incubation time,  $V$  [ $\text{NL}_{\text{CH}_4}/\text{kg}_{\text{VS}}$ ] is the cumulative volume of biomethane at time  $t$  (d),  $V_{\max}$  [ $\text{NL}_{\text{CH}_4}/\text{kg}_{\text{VS}}$ ] is the maximum cumulative volume at the end of the BMP test, and  $k$  ( $\text{d}^{-1}$ ) is the specific rate constant (P. Li et al., 2018). The values of the first-order kinetic constants obtained from the best fit of the data based on the correlation coefficient ( $R^2$ ) values are reported. Non-linear regression was carried out by Scilab (Dassault Systèmes, Vélizy-Villacoublay, France) in order to extract the kinetic parameters ( $k$  and  $V_{\max}$ ) of the first-order model.

#### **IV.2.2.7 Advanced Characterization Techniques**

##### **IV.2.2.7.1 Scanning Electron Microscopy (SEM)—Energy Dispersive Spectroscopy (EDS)**

The surface morphologies of raw and pretreated straw were characterized by SEM using Quanta 250 FEG system (FEI Company, Hillsboro, OR, USA). Before SEM observation, a thin layer of 5 nm thickness of platinum was coated on the samples to have higher resolution and

magnification and to avoid beam damage to sensitive samples. EDS was used for elemental identification on the surface of the straw.

#### **IV.2.2.7.2 Simons' Staining**

Simons' staining was performed according to the modified method proposed by Chandra et al. (2008b). In a preliminary step, DO dye (Pontamine Fast Orange 6RN) was fractionated to recover only the high molecular weight fraction of the commercial product which is a mixture of polydispersed compounds. Therefore, a DO solution (1% w/v) in water was fractionated by ultrafiltration through a 100 K membrane using an Amicon ultrafiltration apparatus (Amicon Inc., Beverly, MA, USA) under a gas pressure of 28 psi. Only the retentate was used in further experiments. To calculate the concentration of DO dye after ultrafiltration, a known volume (1.0 mL) of the solution was dried in a 50°C oven for 5 days and the weight of the solid residue was measured.

To measure the amount of adsorbed dye on the fiber, 20 mg of each wheat straw sample was weighed into a 2 mL Eppendorf tube, and 2 mL of a solution was added. The composition of the solution, for a total volume of 2 mL, was the following: 0.2 mL of PBS solution (phosphate-buffered saline solution, pH 6, 0.3M PO<sub>4</sub>, 1.4 mM NaCl), 0.6 mL of DO solution (10.0 mg/mL), 0.6 mL of DB solution (10.0 mg/mL) and 0.6 mL of water. Tubes were incubated at 70 °C for 6 h, with shaking at 200 rpm. After the incubation period, the tubes were centrifuged at 10,000 rpm for 5 min and 20 µL of the supernatant was diluted to 2 mL, placed in a cuvette, and the absorbance was read on a Libra Biochrom S60 UV–vis spectrophotometer (Biochrom LTD, Cambridge, UK) at 624 and 455 nm. The amount of each dye adsorbed on the fiber (mg dye/g fiber) was determined using the difference in the concentration of the initially added dye and the concentration of the dye in the supernatant—this latter corresponds to the free dye (mg dye/mL solution).

To calculate the corresponding concentration of each dye ( $C_O$  and  $C_B$ ) from the absorbance, two Lambert-Beer law equations (as given in Equation (IV.2-2)) for binary mixture must be solved simultaneously where  $L$  is the pathlength which is represented by the width of the cuvette (cm) and  $\epsilon$  is the extinction coefficient of each component at the respective wavelength. The extinction coefficients for DO and DB dyes were calculated by preparing standard curves of each dye and measuring the slope of their absorbance at 455 and 624 nm. The values calculated and used in this study were  $\epsilon_{DO/455nm} = 25.364$ ,  $\epsilon_{DB/455nm} = 6.255$ ,  $\epsilon_{DO/624nm} = 0.73$ , and  $\epsilon_{DB/624nm} = 65.524 \text{ L g}^{-1} \text{ cm}^{-1}$ .

$$\begin{cases} A_{455\text{nm}} = \varepsilon_{\frac{\text{DO}}{455\text{nm}}} L C_{\text{O}} + \varepsilon_{\frac{\text{DB}}{455\text{nm}}} L C_{\text{B}} \\ A_{624\text{nm}} = \varepsilon_{\frac{\text{DO}}{624\text{nm}}} L C_{\text{O}} + \varepsilon_{\frac{\text{DB}}{624\text{nm}}} L C_{\text{B}} \end{cases} \quad (\text{Eq. IV.2-2})$$

The amount of each dye necessary to reach saturation was previously determined by measuring the dye absorption isotherm for the untreated straw. The ratio of the amount of adsorbed orange dye to the amount of adsorbed blue dye (DO/DB) has been used as an estimation of substrate porosity. In short, the affinity of DO for the straw is likely to be higher when the porosity of the solid is enhanced, thus facilitating the diffusion of the large dye into the pores (Chandra et al., 2008a; Yu and Atalla, 1998).

### IV.2.2.7.3 Infrared Spectroscopy

#### IV.2.2.7.3.1 Diffuse Reflectance (DRIFT)

To perform DRIFT and WAXD characterizations (as explained in Section IV.2.2.7.4), the sample size was further reduced using a blender and sieved (mesh size between 0.355 mm and 0.125 mm).

Infrared spectra for all samples were collected using an FTIR spectrometer (Bruker IFS66V, Bruker, Billerica, MA, USA) equipped with a diffuse reflectance accessory (DRIFT, Collector—Spectratech, Thermo Fisher Scientific, Waltham, MA, USA). The latter is a high-temperature cell equipped with ZnSe windows and with gas inlet and outlet connection. Samples were size reduced and dispersed in diamond powder, with a 1:1 sample/diamond ratio (mg sample/mg diamond). Samples were then placed into a macro cup, ensuring the removal of the excess sample, and smoothening the sample surface. All DRIFT spectra were collected at a set temperature of 50°C and under argon flux, to reduce the spectral contribution of atmospheric carbon dioxide and water vapor. Spectra were obtained from 600 cm<sup>-1</sup> to 4000 cm<sup>-1</sup>, with a scan resolution of 1 cm<sup>-1</sup> and averaging 128 scans/spectrum. The (pseudo) absorbance was obtained by measuring the reflectance of diamond powder as a reference sample, according to the theory developed by Kubelka and Munk (Kubelka, 1948; Sirita et al., 2007).

#### IV.2.2.7.3.2 Attenuated Total Reflectance (ATR)

The presence of various functional groups was assayed using a Nicolet iS5-iD3 ATR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For this, analysis was performed over the spectral range of 4000–400 cm<sup>-1</sup> with 16 scans.



#### IV.2.2.7.4 Wide-Angle X-ray Diffraction Analysis (WAXD)

WAXD measurements were performed using a D8 Discover diffractometer (Bruker, Billerica, MA, USA) with a CuK $\alpha$  radiation ( $\alpha = 1.542 \text{ \AA}$ ) operating at 40 kV and 30 mA. The source slit was 6 mm and the detector slit was 9 mm. The scan was obtained from 10 to 50 degrees  $2\theta$  in 0.25 degrees steps for 1 s per step.

To calculate the crystallinity index (CI) of cellulose, four crystalline peaks (corresponding Miller indices 110,  $1\bar{1}0$ , 102, and 200) in the  $2\theta$  range between 13 and  $25^\circ$  were considered as well as a broad Gaussian amorphous peak. The fitting of the diffraction pattern was performed by a curve-fitting process using Fityk software version 1.3.1 (Ahvenainen et al., 2016a; Murthy and Minor, 1990a; Wojdyr, 2010). The area of the four crystalline peaks ( $A_{cr}$ ) and the total area (crystalline and amorphous peaks,  $A_{sample}$ ) are used to calculate the crystallinity as shown below (Ahvenainen et al., 2016a):

$$CI = \frac{A_{cr}}{A_{sample}} \quad (\text{Eq.IV.2-3})$$

Cellulose microfibril crystallite size is obtained by the width of the diffraction peak associated with the specific reflecting plane (200),  $L_{200}$ , calculated using the Scherrer equation (Cullity and Stock, 2015):

$$L_{200} = \frac{0.9 \lambda}{\beta_{200} \cos \theta} \quad (\text{Eq.IV.2-4})$$

where  $\lambda$  is the X-ray wavelength in  $\text{\AA}$ ,  $\beta_{200}$  is the angular full-width at half maximum intensity (FWHM) in radians of the peak at  $22.0$  radians (corresponding to the 200 Miller indices) and  $\theta$  is the scattering angle.

#### IV.2.2.7.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

Solid-state magic angle spinning nuclear magnetic resonance (MAS NMR) experiments were performed on a Bruker Avance 500 spectrometer (Bruker, Billerica, MA, USA) with 11.7 Tesla magnets and 4 mm zirconia rotors, spinning at the rate of 14 kHz. The resonance frequency of  $^1\text{H}$  and  $^{13}\text{C}$  were 500.16 MHz and 125.78 MHz, respectively. Chemical shifts,  $\delta$ , were reported relative to adamantane powder (38.52 ppm).  $^{13}\text{C}$  quantitative spectra were recorded with the multiple Cross-Polarization (multi-CP) sequence proposed by Johnson and Schmidt-Rohr (2014). Eight CP periods were used, with a length of 1s between each of them. The contact time was 1 ms and the  $\pi/2$  pulse duration for  $^1\text{H}$  and  $^{13}\text{C}$  was 3.1 ms. Recycle delay and height power decoupling were 5 s and 70 kHz, respectively.

Schematic representation of the pretreatment steps followed and the naming of the samples used in the following sections are given in Figure IV-6.

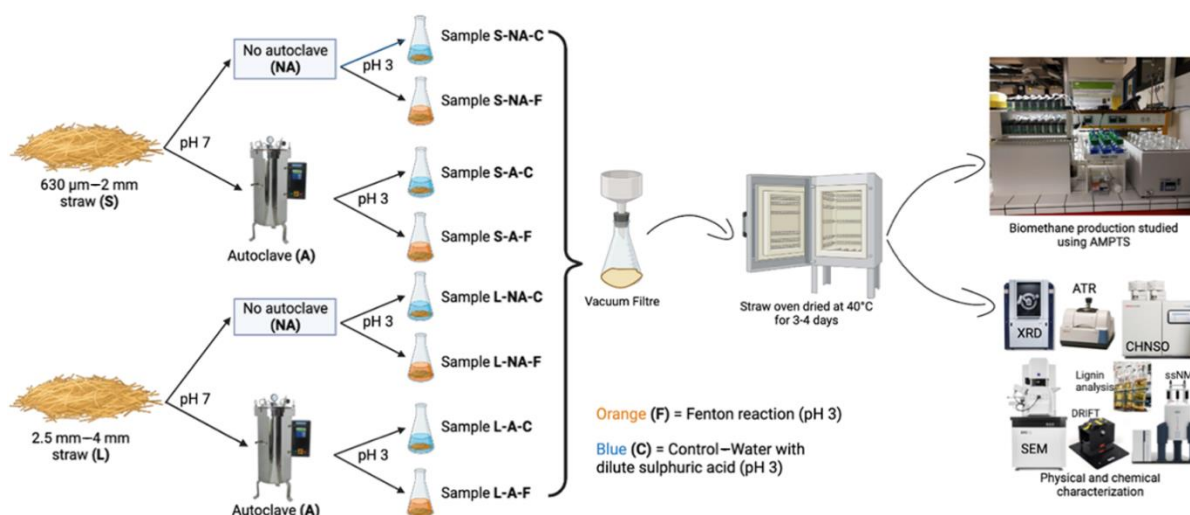


Figure IV-6: Schematic representation of the experimental flow in this study

## IV.2.3 Results

The ground and sieved straw was separated into two size fractions and accordingly pretreated by autoclave and Fenton reaction (see Figure IV-6). On the pretreated samples and the controls for each pretreatment, BMP measurement (as described in IV.2.2.6) was carried out. Characterization using a range of techniques as explained the Sections IV.2.2.5 and IV.2.2.7 was realized in order to understand the changes that the biomass undergoes during the pretreatment. The relevant results will be presented in this chapter.

### IV.2.3.1 Physio-Chemical and Chemical Characterization

#### IV.2.3.1.1 TS, VS, and Elemental Composition

The TS, VS (%TS), and elemental composition are shown in Table IV-4. As observed in the analysis, the biomass contains a higher proportion of C, O, and H contents, which shows the higher energy potential (i.e., calorific value) of straw. Nevertheless, C is very high compared to N, and therefore the C/N ratio is very high for the anaerobic digestion of straw, suggesting a lack of nitrogen source when performing anaerobic digestion. This can be adjusted by co-digestion with nitrogen-rich substrates, such as animal manure (Croce et al., 2016). Using simplified algebraic equations, the theoretical amount of biogas can be calculated from the relative amounts of C, H, N, S, and O. Based on Boyle's formula as given in Achinas and Euverink (2016) (Achinas and Euverink, 2016), a theoretical BMP of  $475 \pm 3 \text{ NL}_{\text{CH}_4}/\text{kg}_{\text{VS}}$  was obtained for control straw samples. However, this calculation takes into account assumptions,

such as the complete conversion of biomass and ideal conditions and it does not consider the energy demand of the microbes or the non-degradable fraction (Achinas and Euverink, 2016).

**Table IV-4:** Total solids (TS), Volatile solids (VS(%TS)), and elemental composition (C, H, N, S, O) of control and pretreated straw

Sample	%TS	VS (%TS)	% C (%TS)	% H (%TS)	% N (%TS)	% S (%TS)	% O (%TS)
S-NA-C	97.8 ± 0.4	98.0 ± 0.0	44.7 ± 0.5	5.8 ± 0.0	0.3 ± 0.0	0.3 ± 0.5	40.4 ± 0.1
S-NA-F	97.0 ± 0.5	98.1 ± 0.3	44.7 ± 0.6	5.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.1	42.7 ± 0.2
S-A-C	96.0 ± 0.5	98.6 ± 0.3	45.1 ± 1.1	5.7 ± 0.1	0.3 ± 0.0	0.4 ± 0.3	43.6 ± 0.5
S-A-F	96.0 ± 0.9	98.6 ± 0.3	43.6 ± 0.6	5.5 ± 0.1	0.3 ± 0.0	0.0 ± 0.0	44.2 ± 0.2
L-NA-C	95.8 ± 0.3	98.7 ± 0.4	44.8 ± 0.1	5.7 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	40.1 ± 0.1
L-NA-F	94.4 ± 0.3	98.6 ± 0.3	44.3 ± 0.5	5.6 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	43.0 ± 0.9
L-A-C	96.2 ± 0.3	99.5 ± 0.0	45.1 ± 1.2	5.7 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	39.6 ± 0.8
L-A-F	94.7 ± 0.3	98.8 ± 0.3	44.2 ± 0.4	5.6 ± 0.1	0.4 ± 0.3	0.3 ± 0.3	43.6 ± 0.6

#### IV.2.3.1.2 Lignin Content Changes in Straw

From Figure IV-7, it can be observed that the proportion of lignin is enhanced after both autoclave or Fenton reaction pretreatment, as well as their combination for small-size particles. This could be due to the lignification of silica bodies (Guerriero et al., 2016) and/or the presence of lignin in stomatal cell walls (Shtein et al., 2017). As can be seen from SEM analysis in Section IV.2.3.3.1, there are more silica bodies and stomata in the pretreated straw of small-size fractions as compared to the large-size fractions. This increase of the lignin proportion was noticed to be even higher for the Fenton reaction-treated samples, showing that the oxidation reactions are not always selective towards lignin degradation (Croce et al., 2016). However, lignin degradation due to the different pretreatment conditions is observed in the large-size fraction. Nevertheless, higher lignin degradation does not always translate into a higher percentage of BMP enhancement (as seen in Section IV.2.3.2). This is because lignin is not detrimental to the further degradation of the biomass during AD since it is known that depolymerization of lignin takes place during this process, and could even be improved in presence of a suitable microbial community (Mulat and Horn, 2018).

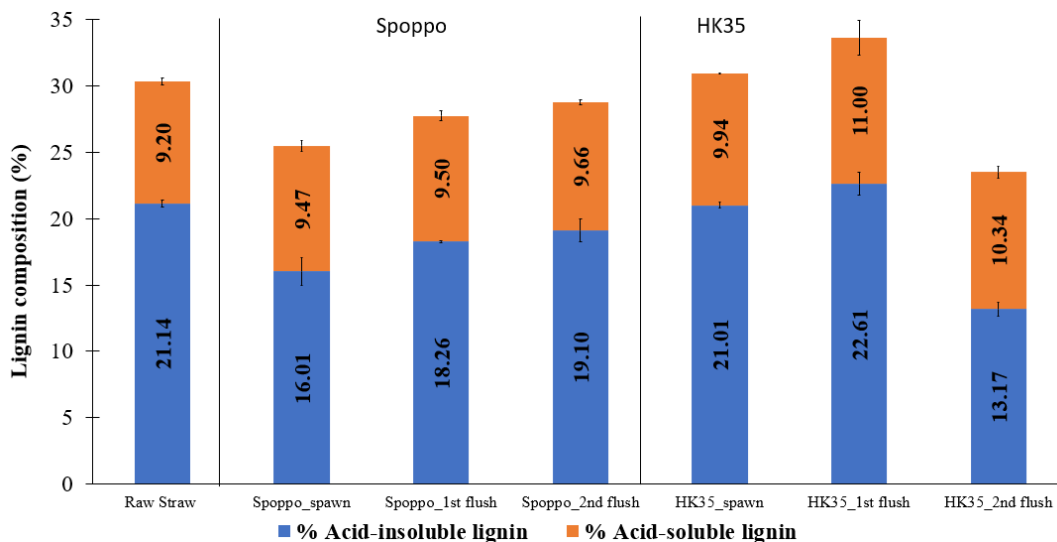


Figure IV-7: Total lignin content (%) as a sum of acid-soluble (ASL) and acid-insoluble (AIL) fractions in pre-treated samples as compared to their corresponding controls

#### IV.2.3.2 Biochemical Methane Potential (BMP)

The cumulative methane production curves obtained from the BMP tests for large and small-size fractions are shown in Figure IV-8 A and B respectively. It can be observed that most of the biomethane was produced in the first 30 days and then the production slowed down. There was no lag phase observed since the inoculum is well adapted to agricultural residues. The total cumulative methane yield after 57 days for all samples is represented in Figure IV-9.

Overall, the cumulative methane yield is significantly higher for the small-size fraction straw whatever the pretreatment as compared to the large-size fraction straw. A maximum increase in methane yield due to particle size reduction was found to be 24.3% ( $p < 0.05$ ) (as shown in Figure IV-9). The hydrolysis rates ( $k$ ) (as seen in Table IV-5) were also significantly increased up to 39% ( $p < 0.05$ ) for Fenton pretreated samples due to size reduction. It can be assumed that the reduced particle size increases the surface area for bio-accessibility of the solid straw, and therefore the methane production rate and final methane yield. This is similar to the findings of Menardo et al. (2012) where the reduction of the particle size of wheat straw from 5 cm to 0.2 cm led to a methane yield increase of 17% (Menardo et al., 2012). However, Dumas et al. (2015) found no change in the methane potential value due to micronization (when the median diameter of wheat straw was reduced from 759 mm to 48 mm). However, they observed an improvement in the biodegradation kinetics when the median diameter of wheat straw was reduced from 759 mm to 200 mm, below which there was no significant increase in the kinetics as well (Dumas et al., 2015).

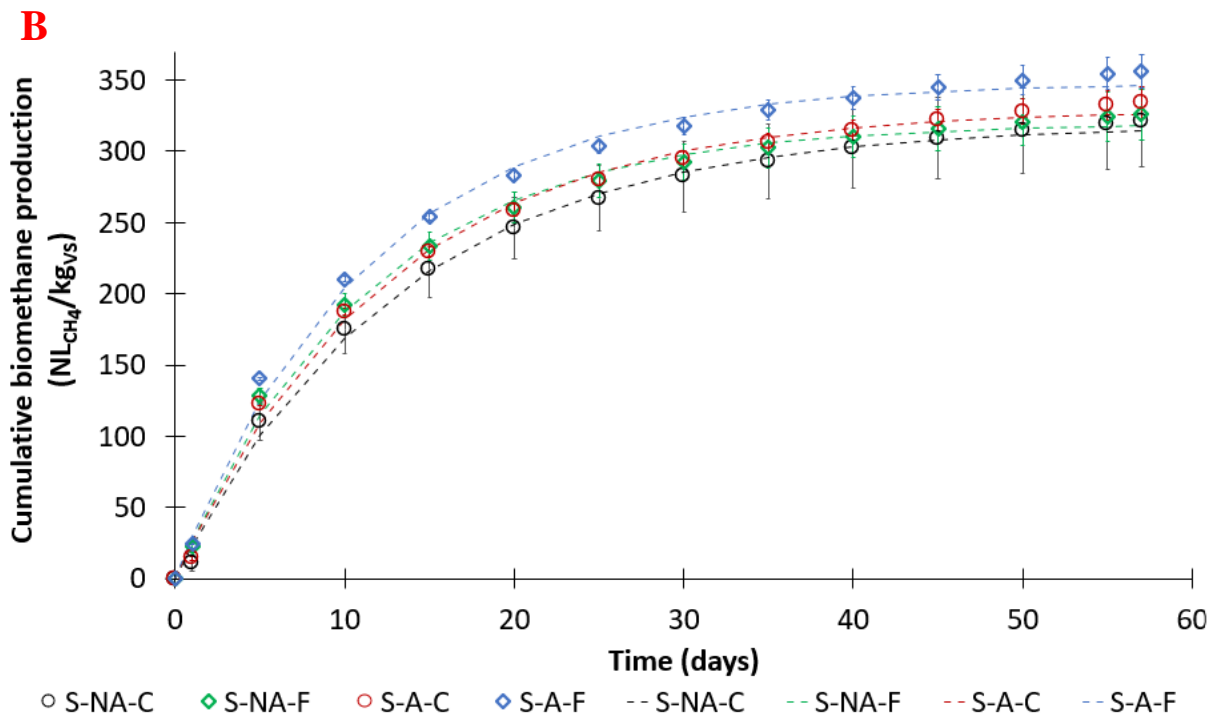
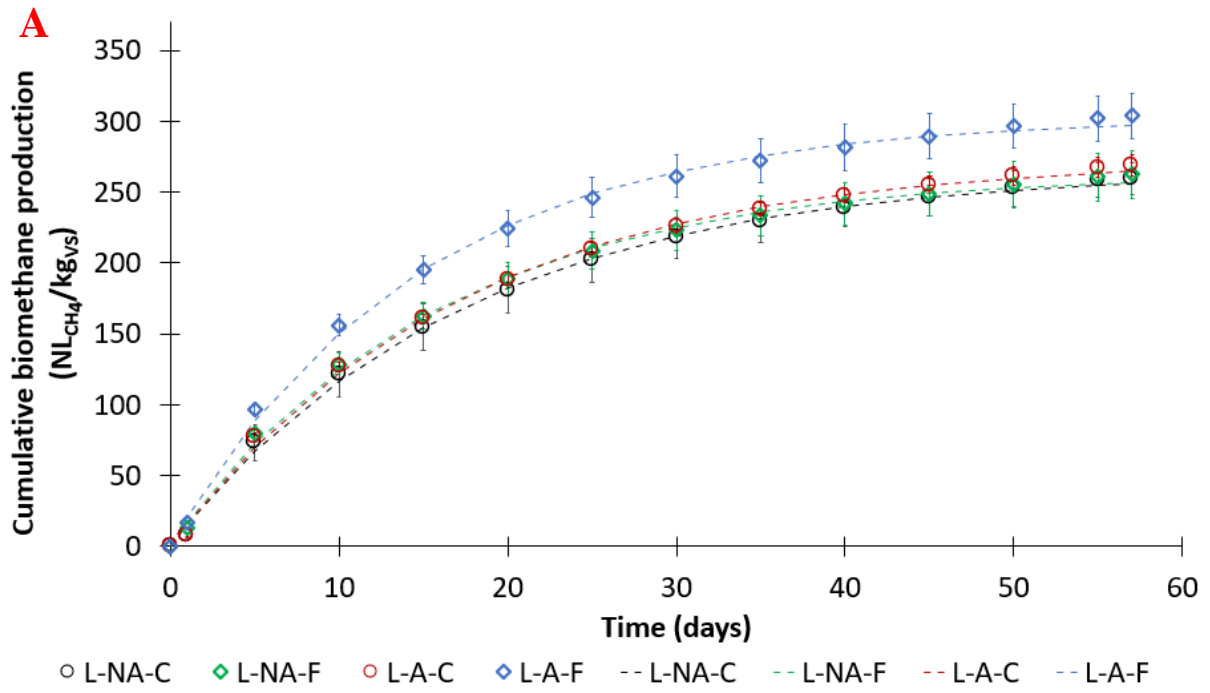


Figure IV-8: Cumulative biomethane production over time for the large-size straw fraction (A) and small-size straw fraction (B). The markers represent the average cumulative biomethane potential (with standard deviations as error bars) and the dotted lines represent the first-order modeled curves.

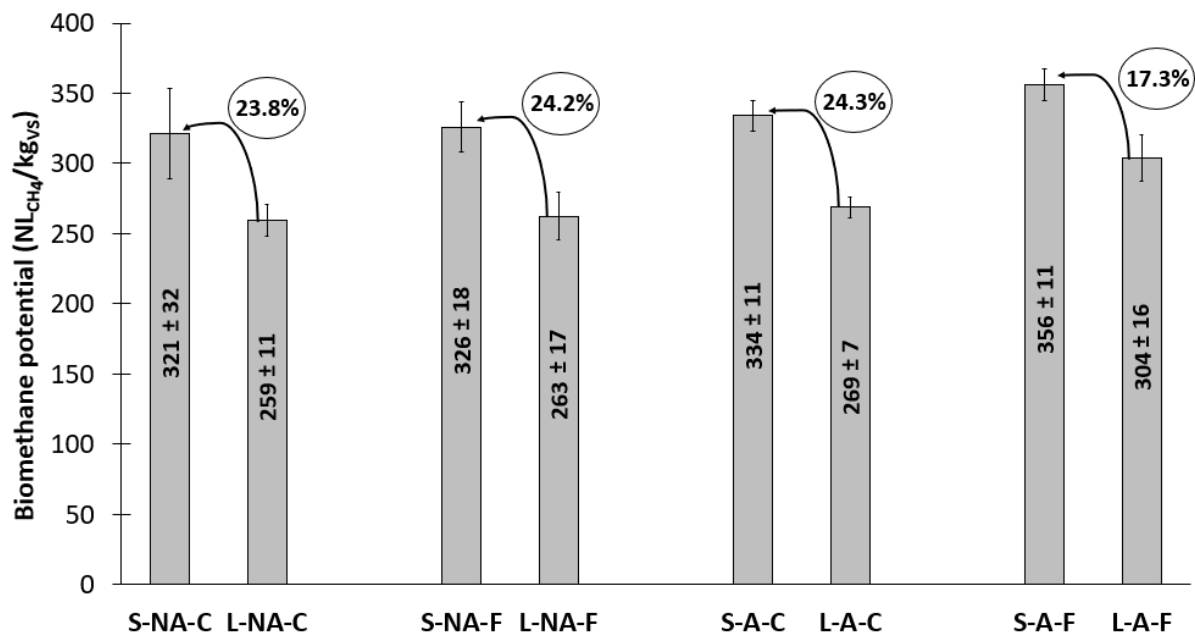


Figure IV-9: Total cumulative methane yield after 57 days

Table IV-5: Parameters of first-order kinetic

Sample	Vmax (NL <sub>CH<sub>4</sub></sub> /kg <sub>vs</sub> )	k (d <sup>-1</sup> )	R <sup>2</sup> (-)
S-NA-C	318 ± 29	0.076 ± 0	0.997
S-NA-F	320 ± 16	0.089 ± 0.002	0.995
S-A-C	329 ± 10	0.081 ± 0.005	0.996
S-A-F	348 ± 10	0.089 ± 0.004	0.994
L-NA-C	266 ± 6	0.058 ± 0.008	0.998
L-NA-F	264 ± 17	0.064 ± 0.002	0.998
L-A-C	274 ± 8	0.059 ± 0.003	0.998
L-A-F	303 ± 17	0.069 ± 0.002	0.997

Autoclaving the straw samples only improved the cumulative methane yield by 4% ( $p = 0.982$ ) for S-A-C and 3.6% ( $p = 0.997$ ) for L-A-C as compared to S-NA-C and L-NA-C respectively. The slight effect of the thermal pretreatment could result from the autoclaving temperature used (121°C), thus preventing any complete hydrolysis (Scherzinger et al., 2020). In the present work, based on Table IV-5, it can be observed that the 1st order rate constant ( $k$ ) increased by 6.6% ( $p = 0.821$ ) and 1.7% ( $p = 1.0$ ) for autoclaved small-size and large-size straw fractions respectively, compared to the control experiment. These results are in the range obtained in another study where the increase in BMP due to autoclaving of food waste was

4.67% (Deepanraj et al., 2017). However, in another study on the effect of autoclaving on biogas production for agricultural biomass (barley straw and sugarcane bagasse), a negative effect on the BMP was observed even though the kinetics was improved while the converse was true for forestry samples (Hazel and Acacia) (Liu et al., 2017). Therefore, the effect of autoclaving on BMP varies with each biomass and the effect of the thermal pretreatment on kinetics has to be taken into account even if there is no significant increase in BMP.

When only Fenton pretreatment was carried out with very low concentrations of  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$ , the cumulative biomethane increase was only 1.5% ( $p = 1.0$ ) and 1.2% ( $p = 1.0$ ) whereas the kinetic constant increase was 17.1% ( $p = 0.017$ ) and 8.5% ( $p = 0.624$ ) for S-NA-F and L-NA-F correspondingly. Therefore, Fenton pretreatment only significantly influenced the kinetics of small-size fractions. However, when the biomass was autoclaved and then followed by Fenton pretreatment, the cumulative biomethane increase was 10.9% ( $p = 0.299$ ) and 17% ( $p = 0.112$ ) respectively for small and large-size fraction straws. The synergy of autoclaving and Fenton pretreatment help to achieve improved hydrolysis and thereby increase the biomethane potential of straw. The combined pretreatment (autoclave and Fenton) improved the kinetic constant ( $k$ ) by 17.1% ( $p = 0.017$ ) and 19% ( $p = 0.067$ ) for small- and large-size fraction straws. Overall, autoclaving combined with low-concentration chemical pretreatment can help considerably enhance the kinetics and the cumulative biomethane potential than individual pretreatments.

### **IV.2.3.3 Physical Characterization of Straw**

#### **IV.2.3.3.1 Surface Morphology Analysis by SEM-EDS**

In Figure IV-10, it can be seen that control samples (S-NA-C and L-NA-C) have a very smooth, rigid surface which is the waxy layer covering the epidermal cells. This organized structure makes it difficult for the enzymes to penetrate and hard to digest (Ma et al., 2022). In the case of small-size straw fraction, the autoclave process and addition of Fenton reagents partially degraded the epidermis and caused the stomata and silica bodies (as depicted in Figure IV-10 S-NA-F, S-A-C, and S-A-F) to be exposed and macro pores to be created. The pores are created due to the dissolution of lignin and hemicellulose as Fenton's reagents preferentially degrade hemicellulose and lignin (J. Li et al., 2019).

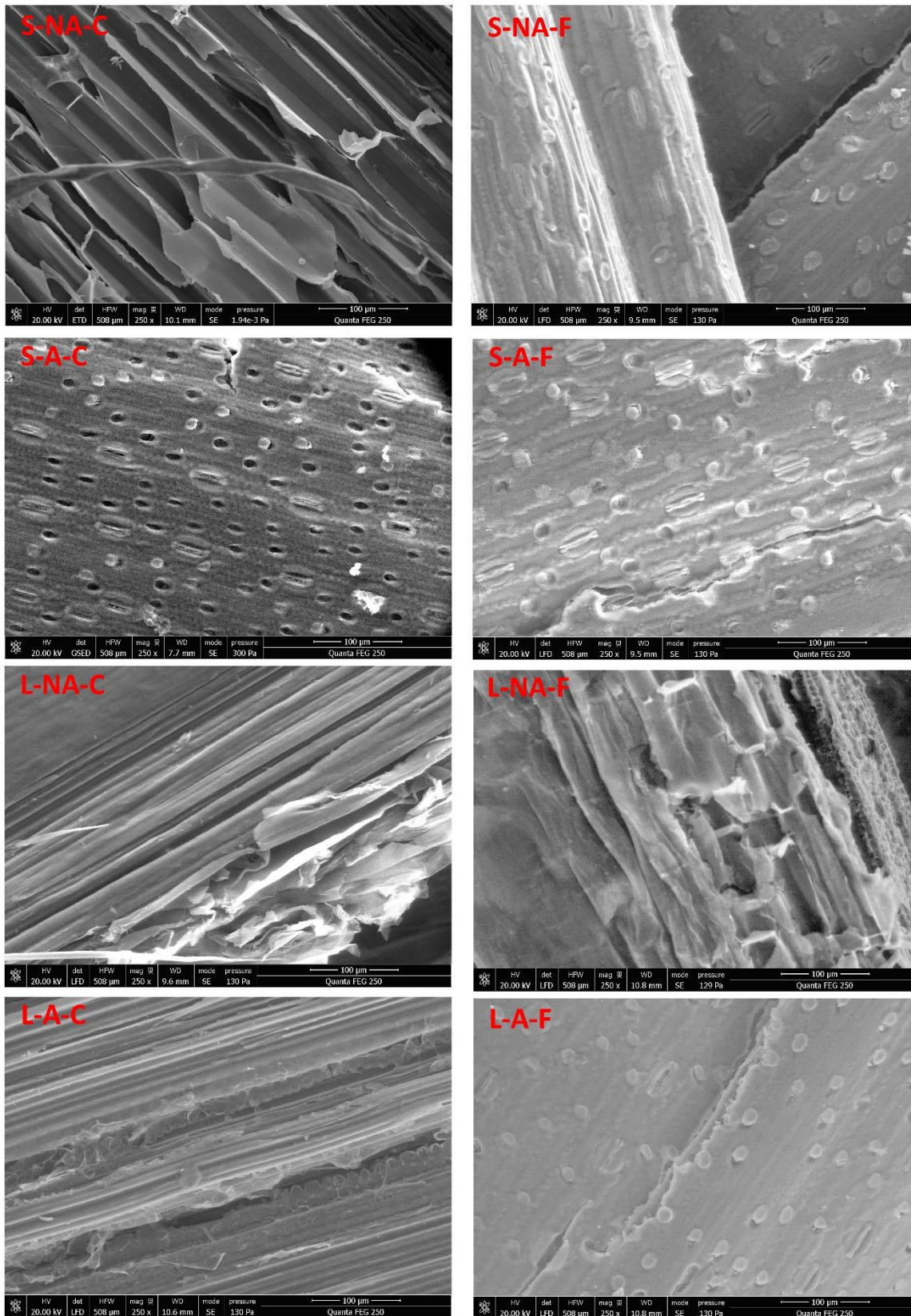


Figure IV-10: SEM images of the control and pretreated straw taken at 250× magnification



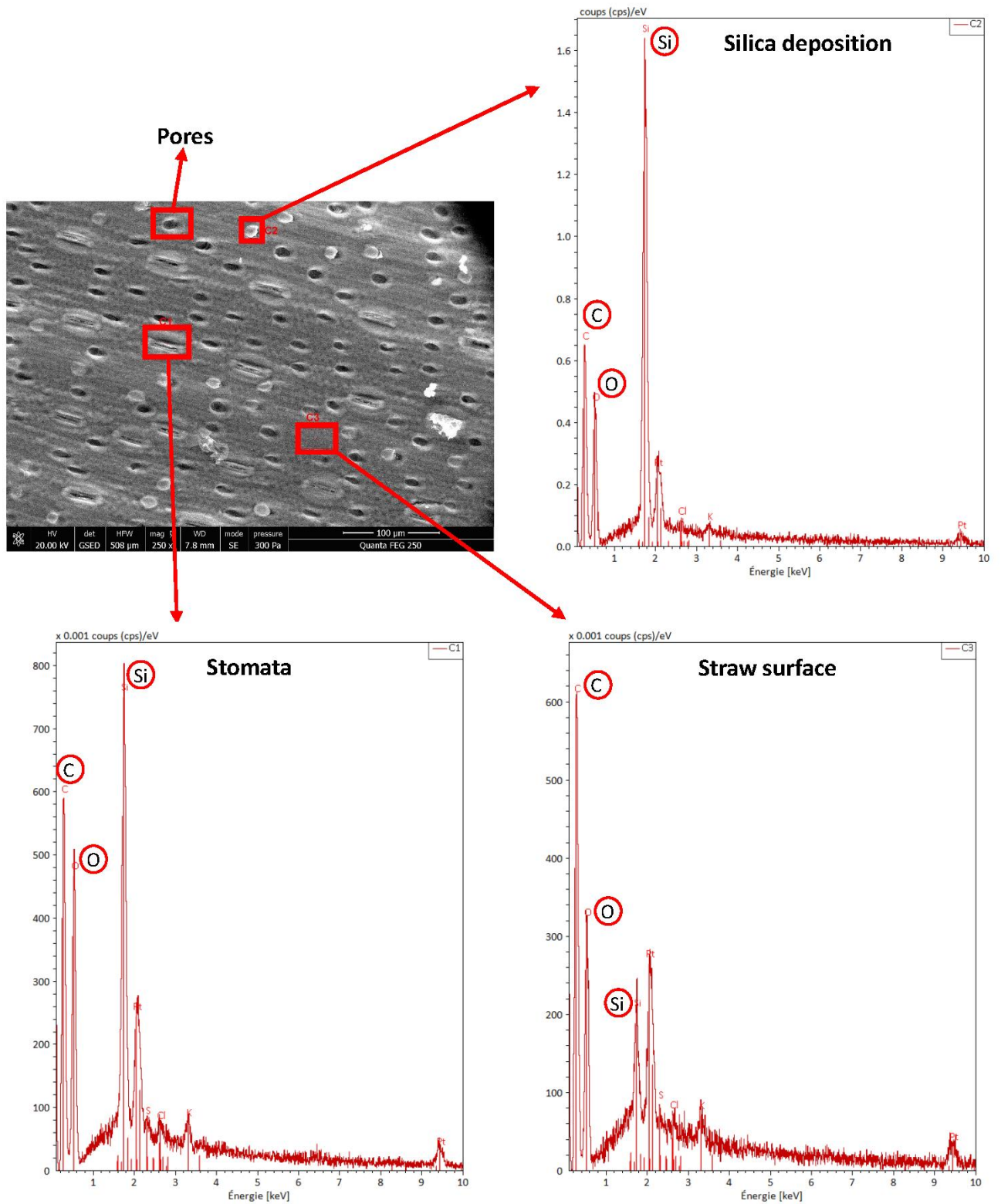


Figure IV-11: SEM-EDS analysis of the S-A-C straw sample

From Figure IV-11, it can be seen that the stomata also have high silica content because they help to reduce transpiration through stomatal pores (Ueno and Agarie, 2005). The higher concentration of platinum in the spectra of EDS is due to the coating used for better resolution of images as mentioned in Section IV.2.2.7.1. As seen in Figure IV-10, for the large-size straw

fractions—L-NA-F and L-A-C, there were not much of structural changes observed as compared to its control (L-NA-C). However, the combined pretreatment of autoclave and Fenton process (L-A-F), did modify the structure and degrade the epidermal cells to expose the stomata and silica bodies. The difference in the surface morphology after different pretreatment conditions between the small size and large size fractions could be due to physical pretreatment. As small-size fraction provides more surface area for further pretreatment to act on, it could cause significant morphological changes to the surface. The more surface area obtained due to size reduction and the creation of pores could have provided more access for microorganisms during anaerobic digestion which resulted in better biogas potential in small-size fractions as compared to large-size fractions. Although SEM analysis is quantitative and obtaining an effective characterization is challenging, it provides an overview of the structure that other analytical techniques do not provide (Karimi and Taherzadeh, 2016). Additional SEM images for each of the conditions studied are provided in the Supplementary Materials (refer to Figure SXI-8 – Figure SXI-15).

#### **IV.2.3.3.2 Porosity Indicator by Simon's Staining**

Simons' staining test is an interesting method used to evaluate any structural variation occurring in biomass ultrastructure upon pretreatment (Simons, 1950). With this test, it is possible to evaluate the variation in the pore size distribution of the lignocellulosic samples (Chandra et al., 2008b, 2008a). For this analysis, two dyes are applied: Direct Blue (DB) 1—a monomeric dye with a molecular diameter of approximately 1 nm—and Direct Orange (DO) 15—a polymeric dye with a molecular diameter of approximately 5 to 36 nm. Due to the difference in size and the higher binding affinity of DO, the DO molecules enter the larger pore and the surface while the DB molecules populate the smaller pores of the biomass (Chandra et al., 2008a; Esteghlalian et al., 2001; Yu et al., 1995). Upon pretreatment of the biomass which might cause an increase in the pore size, DO will enter to a greater extent the enlarged pores. Accordingly, the ratio of the absorbed orange dye to the blue dye (DO/DB) can be used to estimate the pore size distribution of the lignocellulosic samples (Yu and Atalla, 1998). The DO/DB ratio for large and small size fractions upon autoclave and/or Fenton reaction is shown in Figure IV-12.

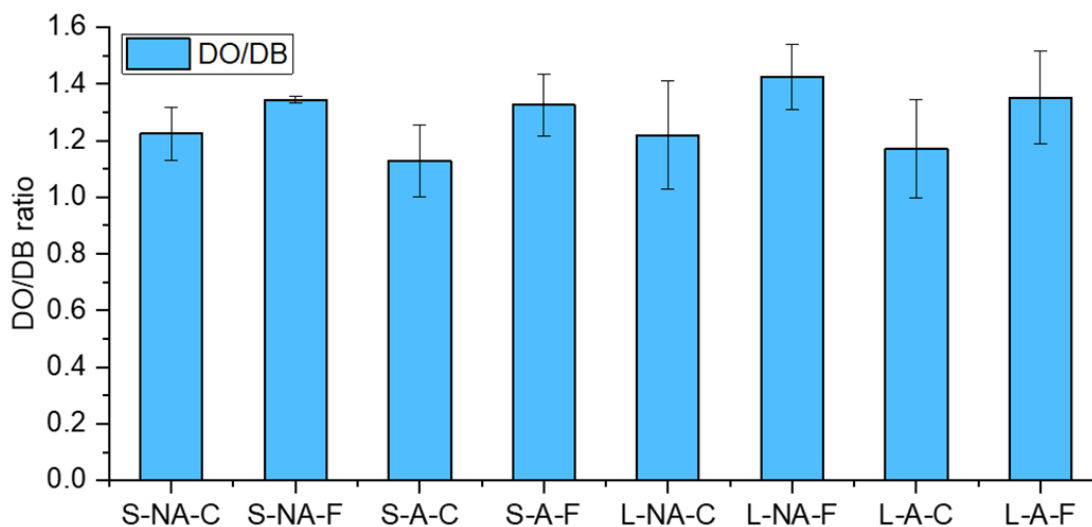


Figure IV-12: Ratio of orange dye to blue dye for control and pretreated straw obtained using Simon's staining technique

Results indicate that the Fenton reaction produces a significant increase in porosity, but the method does not point out any relevant differences between large and small fractions and autoclaved and not autoclaved samples, as confirmed by a three-way ANOVA test (at 0.05 level). However, it must be emphasized that although Simons' staining test is reported to be an indicator of changes in the material in terms of porosity occurring upon chemical and/or physical pretreatments, it remains a qualitative indicator.

#### IV.2.3.3.3 Spectroscopic Analysis of Straw

Changes in the chemical composition of the biomass can occur as a consequence of a pretreatment. Such changes, when significant enough to be detected, can be highlighted by comparing the infrared spectra of lignocellulosic samples before and after the pretreatment and by assigning peaks to the functional groups that are characteristic of each component (cellulose, hemicellulose, and lignin). Thus, the reduction, disappearance, or frequency shift of a peak can be interpreted as a modification or complete/partial removal of the corresponding component (Auxenfans et al., 2017; He et al., 2008; Mamilla et al., 2019; Rodrigues et al., 2022; Stewart et al., 1995; Sun et al., 2014a). Herein we used two infrared spectroscopies, diffuse reflectance (DRIFT) and attenuated total reflectance (ATR) spectroscopy, to try to evidence any spectral variation occurring in straw samples upon autoclaving and/or Fenton reaction.

##### IV.2.3.3.3.1 DRIFT Spectroscopy

DRIFT spectroscopy was mainly used because of the possibility of controlling the experimental conditions (Gogna and Goacher, 2017; Larkin, n.d.) such as the temperature and the moisture of the gas surrounding the sample. To improve the spectra quality some

precautions were taken: the diffusion was enhanced by reducing the size of the samples (particle size ranges between 125 and 355  $\mu\text{m}$ ) (Faix and Böttcher, 1992; Gogna and Goacher, 2017) and the intensity was controlled by conveniently diluting the sample with an inert material, i.e., diamond powder. The spectral contributions of atmospheric carbon dioxide and water vapor were reduced by fluxing an inert gas at 50°C. The DRIFT spectra for all pre-treated samples are shown in Figure IV-13. Spectra obtained were very similar, indicating that either the mild chemical pre-treatment or the autoclave conditions do not induce any significant variations in the chemical composition of the lignocellulosic biomass, or the method is not sensitive enough to detect them.

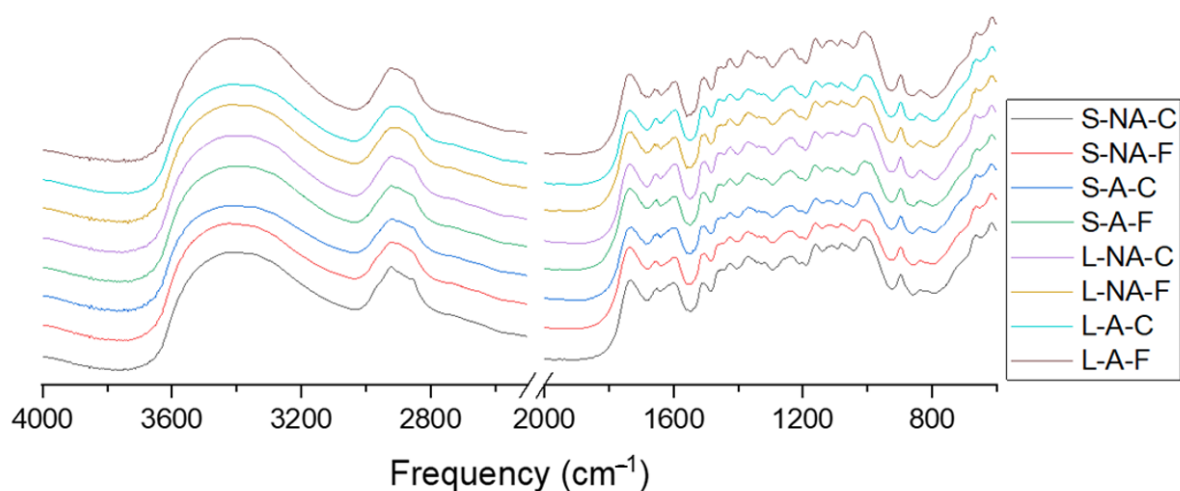


Figure IV-13: DRIFT spectra of control and pretreated samples

#### IV.2.3.3.3.2 ATR Spectroscopy

In addition, ATR spectroscopy was used because this technique is even more rapid and does not require any sample preparation. Moreover, it was suggested as the best mode for Fourier transform infrared (FTIR) analysis of lignocellulose biomass under most circumstances (Gogna and Goacher, 2017). The normalized ATR spectra for all the samples are shown in Figure IV-14. The ATR spectra confirm that no obvious modifications in the FTIR data after the pretreatment are observed.

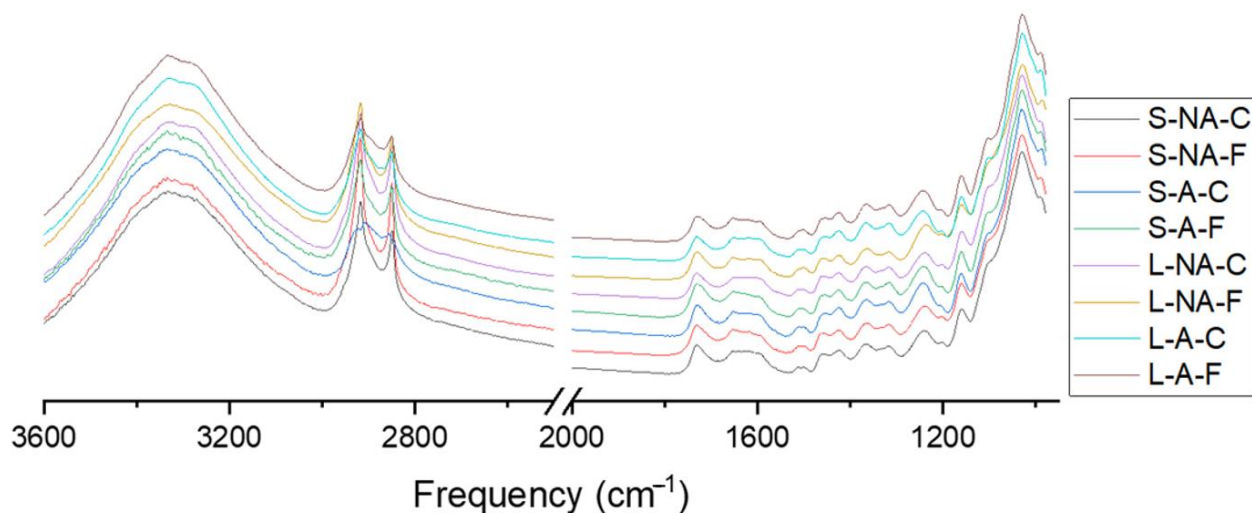


Figure IV-14: Normalized ATR spectra of the control and pretreated samples

However, to accentuate any possible difference between samples, all spectra were also pre-processed and plotted in second derivative mode as reported in Krongtaew et al., (2010) (reported in the Supplementary Materials). Interestingly, when pointing out the most variable regions in the second derivative ATR spectra of the whole set of biomass samples (Figure IV-15 A), and then coming back to the corresponding regions in the ATR spectrum (Figure IV-15 B), some differences become visible in a fingerprint region in the range of 1300–1050  $\text{cm}^{-1}$ . Based on such differences, spectra can be separated into two groups, depending on whether they were autoclaved or not. Namely, the bands at 1235  $\text{cm}^{-1}$  and 1200  $\text{cm}^{-1}$ , both related to the OH out-of-plane vibration mode of cellulose (Schwanninger et al., 2004), are broadened and shifted at lower frequencies for samples not subjected to autoclave. Moreover, the band at 1160  $\text{cm}^{-1}$ , related to the antisymmetric bridge stretching of C-O-C of cellulose and hemicellulose (Stewart et al., 1995), is sharper after the autoclaved samples. Lastly, the intensity of the band at 1100  $\text{cm}^{-1}$ , related to the amorphous cellulose (Nelson and O'Connor, 1964; Stewart et al., 1995), was reduced after the autoclave. These data suggest that slight, also significant, differences in the cellulose and/or hemicellulose can be evidenced by second derivative ATR-FTIR spectra after autoclave.

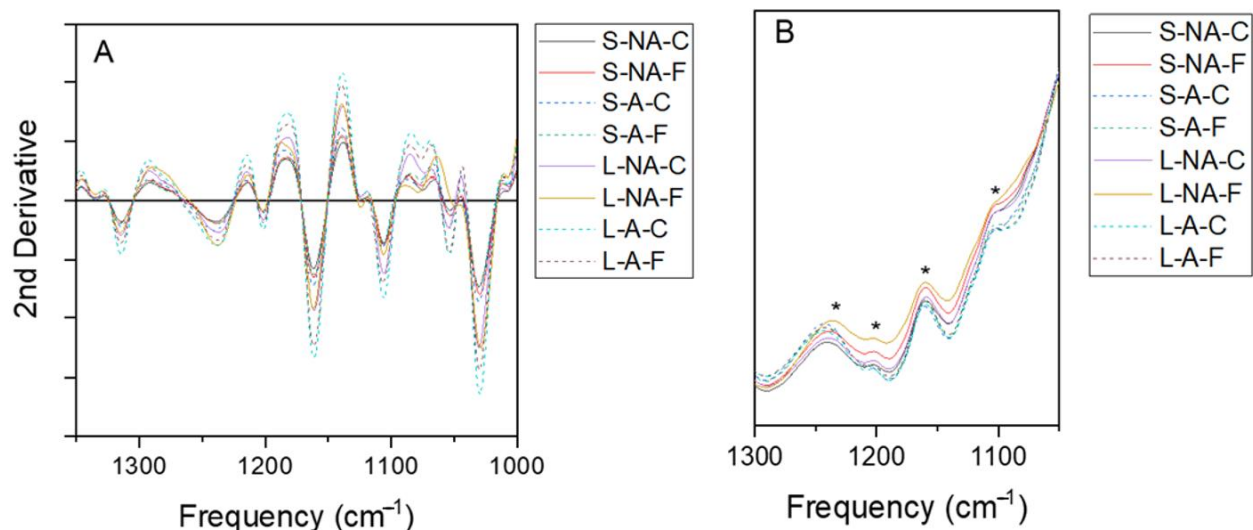


Figure IV-15: (A) Second derivative analysis of ATR spectra in the range 1350 –1000  $\text{cm}^{-1}$ . (B) ATR spectra of controlled and pretreated samples in the range 1300 –1050  $\text{cm}^{-1}$ . The resulting bands modified upon autoclave are indicated with a star (\*)

#### IV.2.3.3.4 Cellulose Crystallinity and Crystallite Size by X-ray Diffraction

The cellulose crystallinity (Crystallinity Index, CI) which is an estimation of the relative amounts of crystalline (ordered) and amorphous (less ordered) part of the biomass is a key parameter often used to characterize the biomass after pretreatment (Park et al., 2010). X-ray diffraction is a widely used technique to determine the CI of the biomass and, in turn, to interpret changes in the solid structure after pretreatments.

Here, the cellulose crystallinity index (CI) was obtained with a curve-fitting process of the X-ray diffraction spectrum into amorphous and crystalline contributions (for more details see Supplementary Materials) (Bernardinelli et al., 2015a; Murthy and Minor, 1990a; Park et al., 2010). This technique (Figure IV-16) shows that CI is around 40% for all straw samples. It can therefore be concluded that the pretreatment conditions studied are not enough drastic to cause conspicuous variations of the cellulose crystallinity.

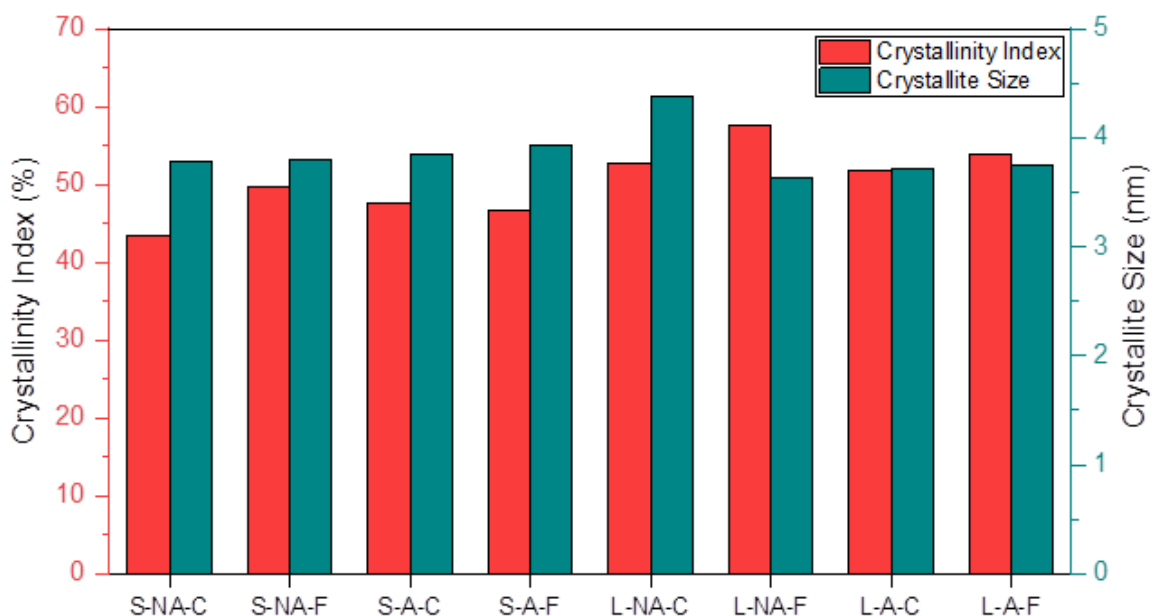


Figure IV-16: Crystallinity index and crystallite size of control and pretreated straw obtained using WAXD

Another important measurable parameter related to cellulose supramolecular structure is its crystallite size. The average crystallite size can be calculated by applying the Scherrer formula, which directly correlates the crystallite size to the width of the crystalline peak (002) (Cullity and Stock, 2015; Park et al., 2010). As shown in Figure IV-16, autoclaving and Fenton pretreatment do not affect the cellulose crystallite size, which remains approximately in the 40 Å-range. As reported by Simons (1950), a crystallite size beyond 40 Å indicates both the coalescence of neighboring microfibrils with the loss of interstitial water and the reduction of amorphous cellulose proportion in the material.

Overall, from XRD data, no significant changes in the CI nor the crystallite size could be evidenced after straw pretreatment. However, it can be concluded that the chosen pretreatment conditions do not lead to a decrease in the amorphous content of the straw, which could be detrimental to its further enzymatic hydrolysis during anaerobic digestion.

#### IV.2.3.3.5 Multi-CP MAS $^{13}\text{C}$ NMR Spectroscopy

Among the techniques used to estimate crystalline index, solid-state  $^{13}\text{C}$  NMR (ssNMR) was reported to be very powerful, leading to more relevant information compared to XRD because it makes it possible to access the CI of cellulose while CI calculated from XRD data reflects the CI of the whole biomass. However, because hemicellulose, lignin, and disordered cellulose domain together contribute toward the amorphous part of the NMR signal, it is necessary to be able to evaluate their relative contributions (Park et al., 2010). A pioneering work from Iversen and co-workers (Larsson et al., 1997a), reported the presence of cellulose

forms with decreasing degrees of crystallinity—namely crystalline, para-crystalline, and amorphous cellulose—and proposed to use of ssNMR to estimate their relative proportions. In addition, the same group demonstrated that ssNMR can discriminate between amorphous cellulose at accessible and at inaccessible fibril surfaces (Wickholm et al., 1998). This method, primarily applied to study pure cellulose, has been later applied to study lignocellulosic biomasses (Bernardinelli et al., 2015a; Foston et al., 2011; Hult et al., 2000).

Here we used the method proposed by Berardinelli et al. (2015) to obtain information on the cellulose crystallinity index of sugarcane bagasse samples by using a spectral editing procedure that removes the lignin signals from the ssNMR spectrum of lignocellulosic biomass (Bernardinelli et al., 2015a). In their work, multi-CP MAS  $^{13}\text{C}$  NMR was preferred to CP MAS since it provides quantitative data (Bernardinelli et al., 2015a; Johnson and Schmidt-Rohr, 2014). In the present work, the lignin spectrum was obtained from the multi-CP MAS  $^{13}\text{C}$  NMR analysis of the precipitated supernatant obtained from an untreated straw sample after a two-step procedure (1%  $\text{H}_2\text{SO}_4$  + 4% NaOH, the detailed procedure is reported in the Supplementary Materials), with the evident advantage of avoiding any chemical treatment that could cause changes in the cellulose crystallinity.

One example of the resulting multi-CP MAS  $^{13}\text{C}$  NMR spectrum of a straw sample after subtraction of the lignin contribution is shown in Figure SXI-5 in the Supplementary Materials. The contributions of hemicellulose and cellulose to the signal are in the 20–180 ppm region, with cellulose peaks in the region between 60 and 120 ppm (inset in Figure IV-17). Beginning from the lower chemical shift ( $\delta$ ), the region between 60 and 70 ppm shows two peaks assigned to the  $\text{C}_6$  carbon of cellulose and hemicellulose, the lower chemical shift being assigned to non-crystalline cellulose and cellulose while the higher chemical shift peaks were assigned to crystalline cellulose. The 70–80 ppm region is a cluster assigned to the  $\text{C}_2$ ,  $\text{C}_3$ , and  $\text{C}_5$  carbons of cellulose and hemicellulose. The regions between 80 and 92 ppm, and between 102 and 108, are associated with the  $\text{C}_4$  and anomeric  $\text{C}_1$  carbons of cellulose and hemicellulose, respectively (Atalla and VanderHart, 1999; Rezende et al., 2011a).



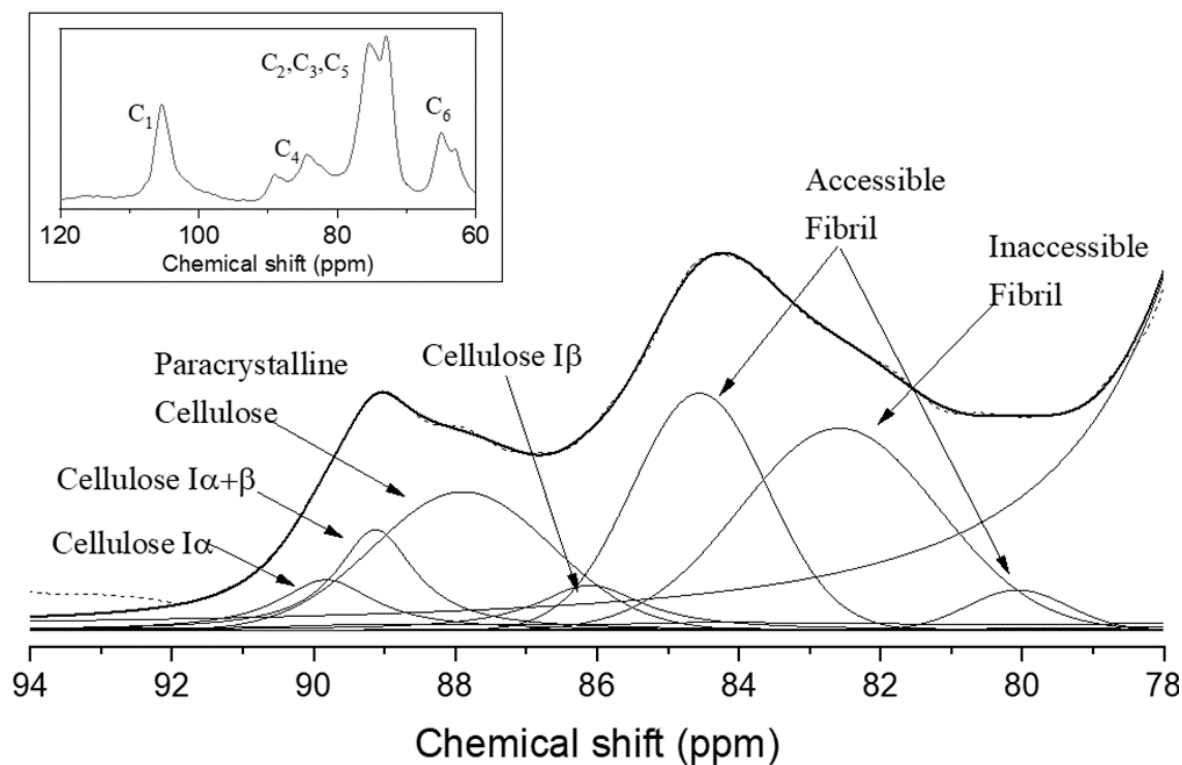


Figure IV-17: Fitting of the C<sub>4</sub>-region S-NA- straw sample). The dotted lines represent the experimental spectrum. The fitted lines and their superposition (ticker line) are shown as solid lines. In the inset on the top left, the NMR spectral region of S-NA-C with the cellulose peaks attribution (Atalla and VanderHart, 1999)

The C<sub>4</sub>-carbon region (Figure IV-17) was used to extract information concerning the cellulose CI of the solid straw samples before and after pretreatment (Bernardinelli et al., 2015a; Ghosh et al., 2019; Wickholm et al., 1998). More in detail, the region typical of the less ordered cellulose (80–85 ppm) is decomposed into three peaks, assigned to accessible ( $\delta$  approximately at 80 and 84.5 ppm) and inaccessible ( $\delta$  approximately at 82.5 ppm) cellulose at the fibril surface. On the other hand, the region typical of the more ordered cellulose (85–92 ppm) contains 4 signals assigned to cellulose crystalline allomorphs I $\alpha$  and I $\beta$  ( $\delta$  approximately at 86, 89, and 90 ppm), and to the cellulose para-crystalline ( $\delta$  approximately at 88 ppm). The spectral fitting of the C<sub>4</sub>-carbon region was performed for all the samples using the model and method reported by Larsson et al. (1997) (Ghosh et al., 2019; Larsson et al., 1997a). The contribution of hemicellulose in the 80–85 ppm range was embedded with the lower chemical shift part of the peak of the C<sub>2</sub>, C<sub>3</sub>, and C<sub>5</sub> carbons cluster. The fitting of the NMR spectrum was performed with the following constraints imposed for all the samples: decomposition into seven peaks with identical maximum positions and FWHM. The result of the fittings of the solid straw samples before and after pretreatment and their assignments are compiled in the Supplementary Materials.

The crystallinity index of cellulose could then be calculated as the ratio of the intensity of the fitted peaks assigned to crystalline cellulose (85–92 ppm) to the intensity of the seven peaks assigned to cellulose in the 80–92 region. The results are shown in Figure IV-18. The cellulose CI for all samples is approximately 40%, which is in agreement with the WAXD analysis (in Section IV.2.3.3.4) and with the data reported in the literature (Liu et al., 2005). The CI remains nearly constant for all the samples, whatever their size or their pre-treatment type, suggesting that the cellulose crystallinity is not altered by the pretreatment.

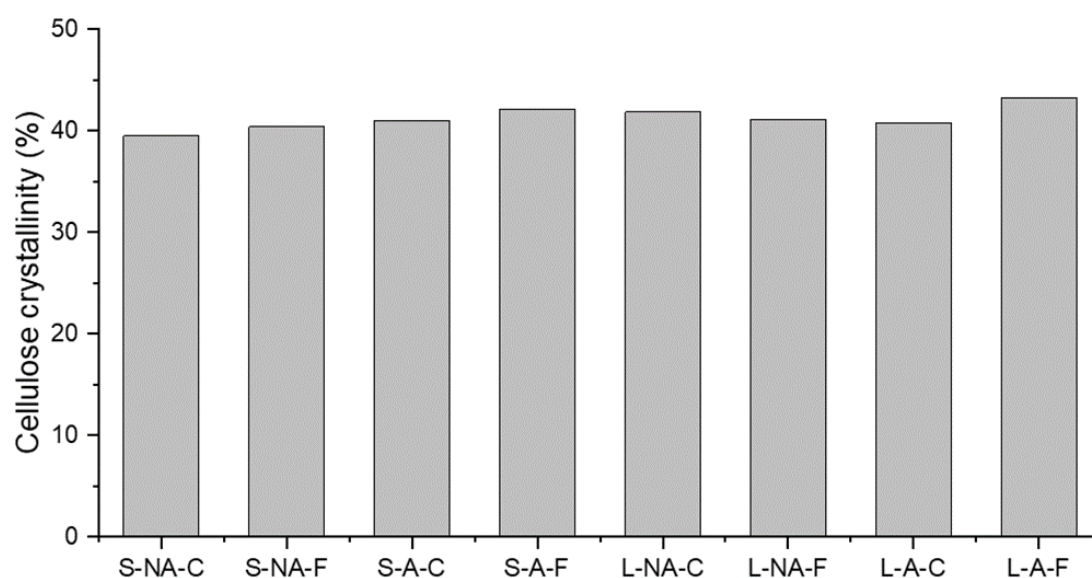


Figure IV-18: Cellulose crystallinity obtained by NMR analysis of the C4-carbon region of cellulose

In addition to the CI calculation, spectral fitting is also useful to estimate the supramolecular structures of cellulose (Chunilall et al., 2010; Hult et al., 2001; Wickholm et al., 1998; Zuckerstätter et al., 2013). Indeed, cellulose is structured in fibrils with a crystalline core surrounded by a non-crystalline cellulose layer. From the fraction  $q$  the intensity of non-crystalline surface cellulose (accessible and inaccessible cellulose) to the total cellulose, and by using a simple fibril model with a square cross-section, the average lateral fibril dimension ( $LFD$ ) can be computed:

$$q = \frac{4n - 4}{n^2} \quad (\text{Eq.IV.2-5})$$

$$LFD = n \times c \quad (\text{Eq.IV.2-6})$$

where  $n$  is the number of cellulose polymers perpendicular to the cross-section along one side of the square fibril cross-section, and  $c$  is a conversion factor of 0.57 nm per cellulose polymer (Hult et al., 2001; Wickholm et al., 1998).

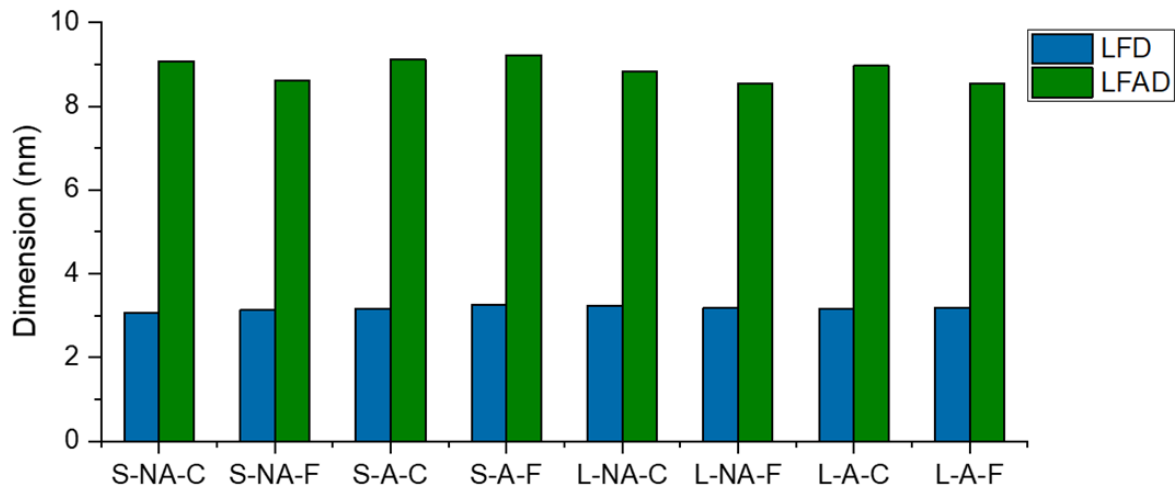


Figure IV-19: Lateral Fibril Dimension (LFD) and Lateral Fibril Aggregate Dimension (LFAD) for the different samples as determined by NMR analysis of the C4-carbon region of cellulose

Moreover, since cellulose fibrils aggregate, only a fraction of the non-crystalline cellulose is accessible to the solvent. Therefore, by considering the fraction  $q$  of only the accessible non-crystalline surface cellulose to the total cellulose, and by using the same fibril model with a square cross-section, the lateral fibril aggregate dimension (LFAD) can also be computed with the same equation. The results of this analysis are shown in Figure IV-19 and indicate that pretreatment conditions have no significant effects on both the fibril dimension (LFD) and the microfibril, or fibril bundle, dimension (LFAD). This was expected since there is no significant variation in the relative amount of accessible and inaccessible cellulose at the fibril surface (See Table S1 in the Supporting Information section). As also reported elsewhere (Sun et al., 2014b), WAXD cellulose crystallite results and NMR LFD results provide a similar trend, even if the WAXD crystallite size results are underestimated in comparison with the NMR LFD.

#### IV.2.4 Discussion and Perspectives

From this study, it is observed that size reduction does not affect the crystallite size, crystallinity index, or porosity. However, there is a significant increase (up to 24%) in the BMP and a drastic increase in the kinetics (up to 39%) due to just a small decrease in the particle size range. Although the methane content of autoclaved or low-concentration Fenton pretreatment varied, they were not significantly different. Nevertheless, the successive pretreatment using autoclave and Fenton increased the kinetics and the BMP. This means that autoclaving plays a key role in enhancing Fenton reactions. This factor should not be ignored when one performs relevant studies as autoclaving is a general practice prior to the study of the effect of biological pretreatment of biomass on biofuel yield. Autoclaving or Fenton pretreatment was shown to

affect the surface of the biomass according to SEM image analysis. Even though there were more morphological changes observed in the small-size fraction samples after autoclaving or Fenton pretreatment, the lignin proportion did not decrease in the samples nor was the cellulose crystallinity affected. Moreover, large-size fractions showed lignin degradation, yet, their methane potential is lower than that of the small-size fraction. These results show that the size reduction provides more surface area for microbial attack during the downstream process which is more important than the lignin degradation efficiency of the pretreatment process. However, it is to be re-iterated that Menardo et al. (2012) could only achieve a 17% increase in biogas yield due to a size reduction from 5 cm to 0.2 mm of wheat straw and Dumas et al. (2015) did not see any increase in BMP due to micronization of wheat straw. Therefore, there is a threshold at which the increase in BMP due to size reduction varies (Dumas et al., 2015; Menardo et al., 2012). This threshold represents the extent to which heat and mass transfer phenomena do not limit the whole biogas production any longer. In this study, combining size reduction, autoclaving, and Fenton pretreatment helped to obtain 75% of the theoretical BMP and significantly increased the kinetics by 53%. When Moset et al. (2018) studied the pretreatment of wheat straw for BMP enhancement, they highlighted that a mild combined mechanical, thermal, and chemical pretreatment allowed them to achieve greater efficiency. Nevertheless, the energy output to input ratio cannot be overlooked for the successful implementation of a pretreatment process.

Uellendahl et al. (2008) studied the energy balance and cost-benefit analysis of biogas production of corn, miscanthus, and willow with and without wet oxidation pretreatment. They concluded that based on the biomass yields obtained in Denmark, the pretreatment of miscanthus and willow shows a positive net ratio of energy output to input as compared to corn while having lower costs for the biomass supply chain and conversion to biogas. This was because the biogas potential of corn did not improve after wet oxidation while there was a significant increase for miscanthus and willow. Moreover, the cost of production of miscanthus and willow is considerably lower as they require less fertilizer and pesticide application than corn, thereby making it a more sustainable choice to use for biogas production (Uellendahl et al., 2008). Therefore, the choice of pretreatment should be based on the capability to reduce the cost of operation, inhibit toxic compound formation, reusability of the chemical used during the process, and which should be environment friendly (Beig et al., 2021).

In recent years, there have been numerous research studies on different pretreatment methods to enhance the digestibility of lignocellulosic material (such as straw and spent coffee). Size reduction is an unavoidable step in preparing the feedstock for the biorefinery process. Many researchers use ground biomass passed through a 2 mm sieve for their pretreatment

studies. In such a practice, the stochastic nature of the grinding and sieving process may cause randomness in results from different research groups. Oyedeji et al. (2020) reviewed the influence of grinding equipment and biomass properties on size reduction and discussed the inconsistency when comparing the results obtained from different analytical methods. There are also various other factors described in these literature studies, such as an increase in the surface area (Dai et al., 2019; Dumas et al., 2015; Kaur, 2022; Sharma et al., 1988), a decrease in the lignin content (Chandler and Jewell, 1980; Xu et al., 2019), reduced crystallinity of cellulose (Hall et al., 2010; Ma et al., 2019; Taherdanak and Zilouei, 2014), and pore size of the substrate in relation to size in enzymes (Meng et al., 2013; Peciulyte et al., 2015; Thompson et al., 1992) which are essential to improve the hydrolysis and thereby increase biofuel yield. However, Ferreira et al. (2014) concluded that cutting the wheat straw to 30–50 mm produced 10.4% higher methane production than grinding the wheat straw to less than 1 mm. Some researchers (Hsu et al., 2010; Yan et al., 2017) have also reported no lignin degradation or increased lignin content after chemical pretreatment and yet improvement in the enzymatic digestibility of the biomass. Contradictory to some literature mentioned before, Grethlein (1985), Kim and Holtzapple (2006), and Pu et al. (2013) have reported that an increased degree of crystallinity because of the removal of amorphous components did not negatively affect enzymatic hydrolysis. Similarly, Zhang and Lynd (2004) also concluded that even if the pore size of the substrate is bigger than that of the enzyme, it is likely that the enzyme is trapped in these pores and therefore, results in a lower hydrolysis rate. This contradictory nature of analytical results makes it difficult to determine which characteristics are most important to declare a pretreatment method as successful (Hendriks and Zeeman, 2009). It is also to be noted that the various characterization techniques used require very few milligrams of the biomass, which is not always representative of the heterogeneous biomass which in turn could lead to biases in the results. One should also pay attention to the particle size used for biomass characterization studies. Standard protocols for lignin analysis (Sluiter et al., 2012) require the biomass particle size to be restricted as deviation from it may result in low or high bias in the results. If analytical protocols call for much size reduction, it could be so overwhelming that the effect of pretreatment would be no more obvious. In this article, we have underscored that the efficiency of pretreatment cannot be explained only using analytical studies but an in-depth analysis of the bioenergy production is needed to validate the choice of pretreatment. The question remains whether the experimentally validated results from pretreatment studies will help to create simulation tools to predict the performance of feedstock. Amon et al. (2007), Dandikas et al. (2014), Thomsen et al. (2014), and Triolo et al. (2011) have all developed models for BMP prediction of lignocellulosic biomass, each based on a component they found

to significantly influence the methane yield but none of them have considered pretreated biomass so far since the correlation is not very direct.

Ultimately, a poor correlation is found between the biogas production and the characterization results despite the various technologies used. The understanding of the mechanism behind the biogas yield change due to pretreatment is still not clear. The authors call for further research into the improvement of current biomass characterization techniques, such as infrared spectrometry, microscopic analysis, NMR, and accessibility studies. Researchers are also encouraged to develop novel surface analytical methods with a multidisciplinary collaboration between surface chemistry and (bio)chemical engineering science.

#### **IV.2.5 Conclusions**

A combination of particle size reduction (<2 mm), autoclaving (121°C at 2 bars for 20 min), and Fenton reaction (1.047 mM of Fe<sup>2+</sup> and 0.1475 M of H<sub>2</sub>O<sub>2</sub>) produced the highest methane potential (356 ± 11 NL<sub>CH<sub>4</sub></sub>/kg<sub>VS</sub>). Grinding of straw contributed to the maximum increase in the biomethane potential. Only Fenton or only the autoclave process improves the kinetics slightly but does not considerably improve the biomethane potential. Combining autoclaving and low-concentration Fenton pretreatment considerably improves the BMP values. Therefore, the synergy of combined pretreatments is better than single pretreatment. The intricacy of the lignocellulosic matrix and heterogeneity in physicochemical composition within the species makes it complex to study.

However, the poor correlation between biogas production and the different physical and chemical biomass characteristics makes it difficult to explain the outcome of various pretreatment methods applied to biomass. Without further improvement and development of analytical techniques, the prediction of the biomethane potential of a feedstock with the aid of pretreatment can only be considered in case-by-case studies.

#### **IV.3 A perspective summary of Chapter IV**

The efficiency of Fenton pretreatment was observed to be dependent on the concentration of Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub>. Lower concentration was enough to cause some structural changes in the biomass and when concentrations of Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> were increased, it lead to radical self-scavenging and side reactions and decreased the degradation rates. It was also observed that the commonly used steps like size reduction and autoclaving contributed to the structural changes in biomass with grinding, in particular, having a greater effect. While the Fenton pretreatment and autoclaving process only contributed to improving the kinetics of

hydrolysis, the physical pretreatment contributed to increasing the overall biomethane potential. Therefore, it is interesting to study the structural changes due to Fenton-like reactions naturally carried out by fungi during the degradation of biomass which is discussed in the following chapter.

## **V Fungal pretreatment of lignocellulosic biomass**

The efficiency of fungal pretreatment depends on the type of fungi, fungal culture conditions, and biomass feedstock. Many studies show the numerous benefits of the *Pleurotus* genus in valuable biotechnological applications and their low-cost nature. Among the *Pleurotus* genus, *Pleurotus ostreatus* is an important strain that has many medicinal and nutritional values (El-Ramady et al., 2022; Onu Olughu et al., 2022). In this chapter, two hybrid strains of *Pleurotus ostreatus*, namely HK35 and Spoppo, used in mushroom cultivation were compared (Chapter V.1). Additionally, pure strains from the mycology bank, namely MUCL 20510, MUCL 28511, and MUCL 29420 were also compared (Chapter V.2). Fungal pretreatment can be carried out in solid-state fermentation (SsF) or submerged fermentation (SmF) conditions. The solid-state condition refers to the cultivation of microorganisms in moist conditions in the absence of any free-flowing liquid, which provides an environment similar to the natural habitat of fungi. The liquid medium in submerged conditions, on the other hand, provides a more uniform environment for the fungi. Both cultivation methods have their advantages and disadvantages (Onu Olughu et al., 2022; Tian et al., 2012). In this chapter, mushroom cultivation which always occurs in solid-state conditions (Chapter V.1), and fungal pretreatment in submerged conditions (Chapter V.2) are studied.

### **V.1 Utilization of spent mushroom substrate for biogas production**

#### **V.1.1 Introduction**

Agricultural residues like straw are majorly used as substrates for mushroom production. In the last decade, the edible mushroom industry has developed rapidly worldwide owing to its nutritional content and unique flavor. Globally, over 6 million tons of mushrooms are produced annually. However, the production of one kg of mushroom on average produces 5 kg of spent mushroom substrate (SMS). SMS consists mostly of the disintegrated substrate along with the fungal mycelium left over after harvesting mushrooms. The management of SMS poses a great challenge for the mushroom industry since improper disposal causes environmental pollution such as water and soil contamination. Therefore, within the framework of “waste-to-energy”, SMS serves as a cheap and abundant feedstock for biogas production. By integrating the mushroom and biogas production, food and energy needs can be satisfied, while improving the overall economy for the mushroom industry (Leong et al., 2022).

Oyster mushrooms, *Pleurotus ostreatus*, are the third most commonly cultivated mushrooms in the world. Oyster mushrooms grow well on straw and are cultivated on pasteurized straw. The intentionally inoculated mycelium on pasteurized straw is called spawn.



The spawn is then let to incubate in controlled temperature and humidity until it has colonized all the new substrates. Further, lowering the temperature and increasing oxygen levels, encourages mushroom formation. Each cycle of growth of mushrooms takes about 2-3 weeks and a crop or harvest of mushrooms is called a flush (Cornell Small Farms). The mushroom industry can utilize the substrate only for about two flushes because the quantity and quality of mushrooms produced decrease beyond this number of harvesting cycles.

## V.1.2 Materials and Methods

### V.1.2.1 Spent mushroom wheat straw

Spent mushroom wheat straw (SMWS) was obtained from La Révolution Champignon, Lille, France (currently called Winergies, Belgium). They provided SMWS samples from two different strains of oyster mushroom, namely Spoppo, and HK35. For each strain, they provided substrate just inoculated with the mycelium, referred to as spawn hereafter. They also provided SMWS obtained after the first flush and after the second flush. The details of the samples received are shown in Table V-1. The samples were studied in comparison to unautoclaved straw (obtained from Hamiform in early 2019) which is called raw straw in this chapter.

**Table V-1:** Details of SMWS samples received from La Révolution Champignon

Sample Name	Substrate	Pleurotus Strain	Flush cycle	Received on
Spoppo_spawn	Wheat Straw	Spoppo	0	23/04/21
Spoppo_1 <sup>st</sup> flush	Wheat Straw	Spoppo	1	23/04/21
Spoppo_2 <sup>nd</sup> flush	Wheat Straw	Spoppo	2	28/05/21
HK35_spawn	Wheat Straw	HK35	0	28/05/21
HK35_1 <sup>st</sup> flush	Wheat Straw	HK35	1	28/05/21
HK35_2 <sup>nd</sup> flush	Wheat Straw	HK35	2	23/04/21

### V.1.2.2 Methodology

#### V.1.2.2.1 Strain isolation and microscopy

The two strains were isolated from the corresponding spawn samples. A small piece of straw with the fungal mycelium was let to grow onto a solid MA1 agar medium (Malt extract (10 g) and Agar (15 g) in 1 L of demineralized water) for 2-3 weeks. A small portion of the fungal mycelia was cut, transferred, and grown on MA1 with chloramphenicol to remove any bacterial contamination. Then again transferred onto solid MA1 plates and stored at 4°C. The strains were then subcultured on a solid medium every two months. 1 mL of sterile demineralized water with 0.1% Tween 80 was used to loosen the mycelia from the agar medium and viewed using an Olympus BX60 microscope attached to an Infinity 3 camera (Lumenera, Ottawa, ON, Canada).

#### **V.1.2.2.2 Characterization of Spent mushroom substrate**

TS, VS, elemental composition, and lignin composition of the substrates were determined in triplicates as mentioned in sections IV.2.2.5.1, IV.2.2.5.2, and IV.2.2.5.3 respectively. For elemental composition and lignin composition, the SMWS samples were dried at 40°C for 3 – 4 days before the analysis.

#### **V.1.2.3 Wide angle X-ray Diffraction (WAXD)**

To perform WAXD characterizations (as explained in Section IV.2.2.7.4), the sample size was further reduced using a blender and sieved (mesh size between 0.355 mm and 0.125 mm). The analysis was performed with the same parameters as described in section IV.2.2.7.4.

##### **V.1.2.3.1 Scanning Electron Microscopy**

The surface morphology of the substrates was viewed using scanning electron microscopy (Quanta 250 FEG system (FEI Company, Hillsboro, OR, USA)). Before SEM observation, a thin layer of 5 nm thickness of platinum was coated on the samples to have higher resolution and magnification and to avoid beam damage to sensitive samples.

##### **V.1.2.3.2 Biomethane potential tests**

The inoculum for the BMP study was obtained from a digester at a municipal wastewater treatment plant in Noyon, France. The inoculum was sieved through a 1 mm sieve and used. The inoculum had a TS of  $2.96 \pm 0.01$  % and VS of  $69.07 \pm 0.14$  % and was used as a negative control. Cellulose along with the inoculum was run as a positive control. The study was conducted in triplicates for the raw straw and SMWS samples as mentioned in IV.2.2.6.1. All the substrates were ground using a laboratory blender and sieved through a 2 mm mesh-size sieve and then again through 630  $\mu\text{m}$  to recover the retentate. This provided a more homogenous size of feedstock for biogas production. The AMPTS system was set up according to the method used in section IV.2.2.6.

### **V.1.3 Results and Discussion**

#### **V.1.3.1 Strains morphology**

Typical hyphal features such as cell walls, cross walls or septa, and vacuoles are seen with the ordinary light microscope in Figure V-1 (A, B). HK35 was the first commercial hybrid *Pleurotus* strain to be produced which had features such as high yielding, and tolerant to a range of growing conditions. Therefore, it is easy to crop all around the year and produces good quality even after several flushes. Spoppo strain has HK35 in its lineage and has the huge

advantage of not producing spores. This helps to prevent the spread of infection by spores amongst the mushroom pickers (Sylvan).

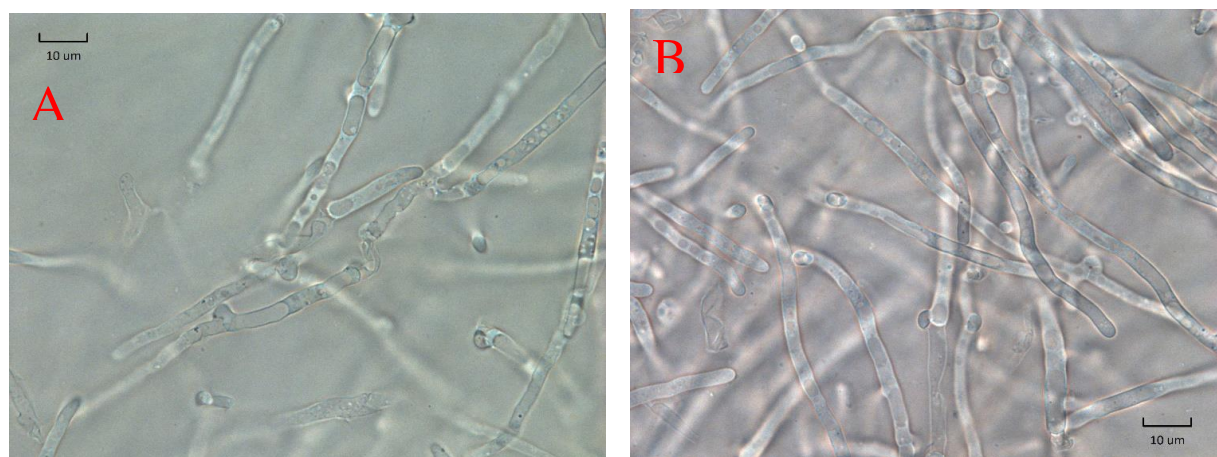


Figure V-1: Strain morphology as viewed under light microscope at 100x magnification of (A) HK35 and (B) Spoppo

### V.1.3.2 Characterization of spent mushroom substrate

Table V-2: Characterization of spent mushroom substrate

Sample	%TS	VS (%TS)	% C (%TS)	% H (%TS)	% N (%TS)	% S (%TS)	% O (%TS)
Raw Straw	93.62 ± 0.21	93.43 ± 0.40	42.87 ± 0.01	5.57 ± 0.03	0.62 ± 0.02	0 ± 0	34.68 ± 1.12
Spoppo_spawn	26.46 ± 1.20	88.81 ± 0.10	38.85 ± 0.22	5.06 ± 0.10	1.04 ± 0.11	1.50 ± 0.13	33.24 ± 0.59
Spoppo_1 <sup>st</sup> flush	32.26 ± 0.51	88.94 ± 1.59	38.11 ± 0.32	5.12 ± 0.06	1.20 ± 0.17	1.03 ± 0.06	33.66 ± 0.69
Spoppo_2 <sup>nd</sup> flush	28.03 ± 1.18	85.29 ± 0.98	35.70 ± 0.09	4.80 ± 0.01	1.05 ± 0.01	1.68 ± 0.07	33.96 ± 0.16
HK35_spawn	24.49 ± 0.53	93.33 ± 0.09	39.70 ± 0.37	5.35 ± 0.03	1.24 ± 0.09	0.38 ± 0.02	35.39 ± 0.36
HK35_1 <sup>st</sup> flush	32.41 ± 0.92	92.43 ± 0.92	40.30 ± 0.50	5.33 ± 0.14	0.99 ± 0.11	0.32 ± 0.01	35.56 ± 1.05
HK35_2 <sup>nd</sup> flush	32.10 ± 1.16	90.50 ± 1.76	37.02 ± 0.27	4.94 ± 0.04	1.04 ± 0.02	0.65 ± 0.01	33.54 ± 0.57

The %TS, %VS, and elemental composition of the raw straw and SMWS are shown in Table V-2. Due to the higher moisture content needed for the inoculation of the spawn and the growth of mushrooms, the %TS is lower in SMWS samples compared to raw straw. Since straw has a very low N content, it is a good substrate as low N content is an important factor for the growth and cultivation of mushrooms. The protein content in the fruiting body of mushrooms depends on the C/N ratio of substrates (El-Ramady et al., 2022). From Table V-2, it can be observed that the percentage of nitrogen in SMWS samples ( $\geq 1\%$ ) as compared to raw straw ( $0.62 \pm 0.02\%$ ) increased slightly. Vane et al. (2001) explained that this increase in nitrogen was due to the incorporation of fungal mycelium in the cell walls of the straw which was detected using  $^{13}\text{C}$  NMR as fungal proteins and lipids. Although nitrogen content increased, it was relatively lower than carbon, hydrogen, and oxygen contents. The % carbon decreased from  $42.87 \pm 0.01\%$  in the raw straw to 35 -37% in 2<sup>nd</sup> flush samples of both strains. This reduction of % carbon is partly due to the mineralization of organic matter. This contributes to a decrease in the C/N ratio. The C/N ratio plays a major role in methane production, the optimal ratio being between 20 and 35 (Bisaria et al., 1990). The raw straw had a C/N ratio of 69 which was reduced to 32 - 41 for SMWS samples. This shows that SMWS samples are good substrates for the AD process and the process can be optimized further by the addition of organic or inorganic nitrogen supplements during the digestion process.

### **V.1.3.3 Lignin Composition**

300 mg of the sample is hydrolyzed with 72% sulphuric acid to determine the relative percentage of lignin in the sample and the lignin composition according to the NREL method (Sluiter et al., 2012). Non-systematic variations in the lignin content were observed among two strains as seen in Figure V-2. The increase in lignin content in 1<sup>st</sup> flush samples as compared to the spawn in both the strains, could be that fungi degrade other parts of the LCB such as cellulose and hemicellulose first. Thus, this results in a relative increase in the lignin proportion. Once these components which are easier to assimilate are less, then fungi start to attack the lignin as a carbon source. The hypothesis that HK35 degrades rapidly the other components and then lignin as compared to Spoppo, could be the reason for the higher lignin degradation of 22.5% in HK35\_2<sup>nd</sup> flush sample.

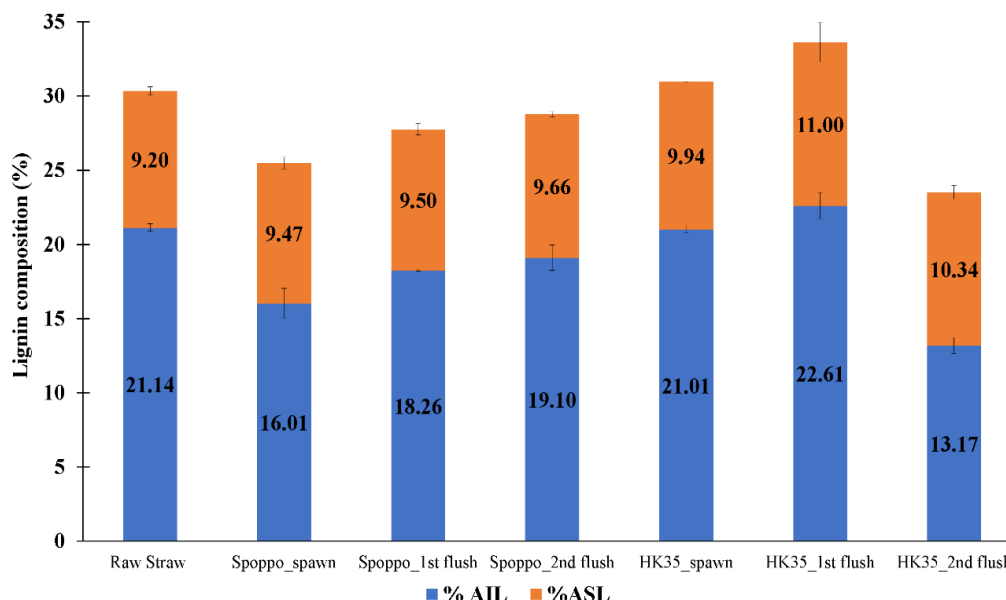


Figure V-2: Lignin composition in terms of acid-insoluble lignin (AIL) and acid-soluble lignin (ASL)

Using solid-state NMR, the change in lignin content of wheat straw during the growth of *Pleurotus ostreatus* was studied by Vane et al. (2001). Even though there was a decrease in the S/G (syringyl to guaiacyl units) ratio of the wheat straw, there was no overall loss of lignin with fungal degradation even after 63 days of growth. Rather, rapid degradation of the amorphous noncellulosic polysaccharides in wheat straw was reported (Vane et al., 2001).

#### V.1.3.4 Crystallinity Index and crystallite size by WAXD

X-ray diffraction was used to determine the crystallinity index (CI) and crystallite size of the biomass which helps to interpret changes in the biomass after pretreatments. The CI is an estimation of the relative amounts of crystalline (ordered) and amorphous (less ordered) states (Park et al., 2010). Amorphous regions are easier to degrade than crystalline regions. It was observed that in the HK35 SMWS, the percentage of the amorphous region decreased from 63% in the spawn sample to 51% after the 1<sup>st</sup> flush with a further reduction to 49% in the 2<sup>nd</sup> flush sample. This increased the crystallinity index from 37.2% (spawn) to 49% (1<sup>st</sup> flush) to 50.7% (2<sup>nd</sup> flush) among the HK35 samples (Figure V-3). Whereas, the amorphous region content of the Spoppo\_spawn sample was 58.5% and only slightly decreased in the 1<sup>st</sup> flush and 2<sup>nd</sup> flush samples. It was around 54% which was similar to raw straw. As a result, the crystallinity index only increased from 41.5% in the spawn sample to 45.6% in the samples after the two rounds of harvest. Vane et al. (2001) also observed a rapid degradation of amorphous noncellulosic polysaccharides and the persistence of relatively crystalline cellulose in fungal-degraded residues. The difference between the two strains could be explained as HK35 degrading only the easily accessible amorphous polysaccharides and then proceeding to

attack lignin (as seen with 22.5% lignin degradation in HK35\_2<sup>nd</sup> flush sample in Figure V-2) whereas Spoppo is slower to attack both polysaccharides and lignin, probably due to slower growth rate. Since we have no particular information from the industry about the fruiting efficiency or growth rate of the two strains, we cannot reach decisive conclusions.

Another important measurable parameter related to cellulose supramolecular structure is its crystallite size. As reported by Simons (1950), a crystallite size beyond 4 nm indicates both the coalescence of neighboring microfibrils with the loss of interstitial water and the reduction of amorphous cellulose proportion in the material. Only the Spoppo\_2<sup>nd</sup> flush sample had a 4.1 nm crystallite size (Figure V-3). Therefore, it is clear that the Spoppo and HK35 attack the amorphous polysaccharides in different ways.

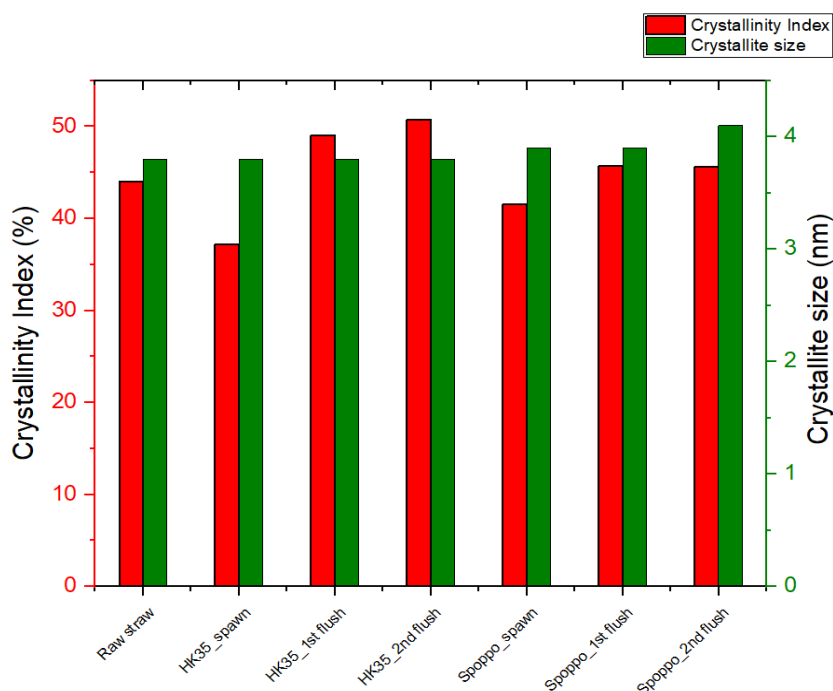


Figure V-3: Crystallinity index and crystallite size of SWMS substrates obtained using WAXD

### V.1.3.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was carried out to visualize the potential structural changes in wheat straw (WS). The raw straw shown in Figure V-4 (A-B), exhibits the smooth, intact, and inaccessible structure of untreated wheat straw. In the HK35 strain SWMS, spawn samples (Figure V-5 (A-B)) show the complete incorporation of the fungal mycelium on wheat straw. After the 1<sup>st</sup> flush (Figure V-5 (C-D)), the SWMS seems to be completely perforated. This is because of the dissolution of lignin and hemicellulose due to the fungal action. After the 2<sup>nd</sup> flush with HK35 strain (Figure V-5 (E-F)), complete structural modification is seen which could provide more surface area for the penetration of enzymes to enhance the saccharification process. This structural modification in the HK35\_2<sup>nd</sup> flush sample has exposed the silica

content present in the cell wall. From SEM analysis, it could be proposed that HK35\_2<sup>nd</sup> flush showed enhanced WS surface depolymerization which also correlates with the low lignin content and more amorphous polysaccharides degradation as observed in section V.1.3.3 and section V.1.3.4. Whereas, poration in the wheat straw along with exposed silica content is already observed in the spawn samples (Figure V-6 (A-B)) of the Spoppo strain. This could be due to various reasons such as a different batch of wheat straw, the extent of pasteurization, and the number of days after inoculation of the strain. Even in the Spoppo strain after 1<sup>st</sup> flush (Figure V-6 (C-D)) , there is no substantial difference in the amount of poration as compared to the spawn samples. However, straw after 2<sup>nd</sup> flush of the Spoppo strain (Figure V-6 (E-F)) has no visible poration but the collapse of the pores and coalescence of microfibrils can be confirmed through the increase in crystallite size beyond 4 nm (Figure V-3). Therefore, the mode of action of Spoppo and HK35 to degrade biomass seems to vary quite a bit.

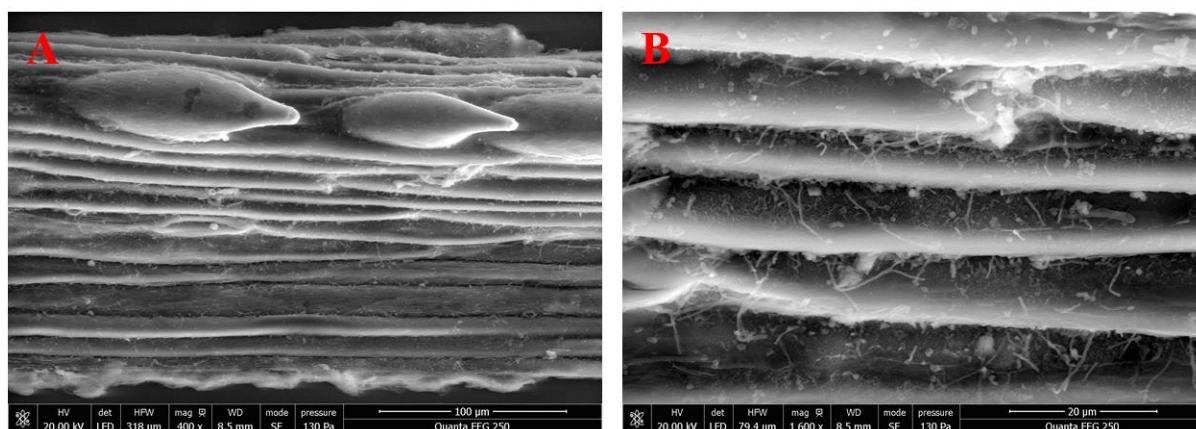


Figure V-4: SEM image of raw straw taken at (A) 400x magnification and (B) 1600x magnification

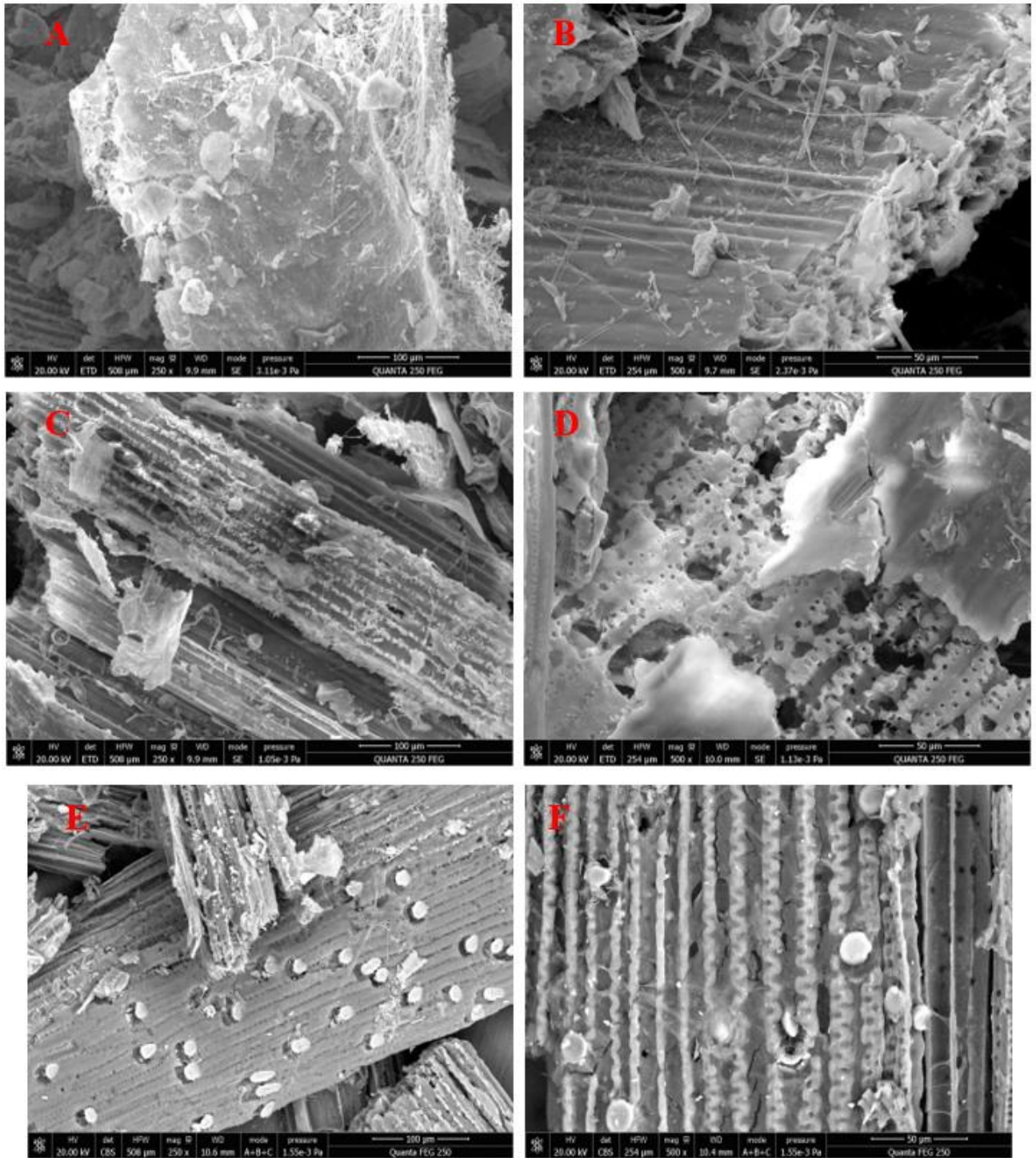


Figure V-5: SEM images of HK35 strain SMWS (A-B) spawn sample, (C-D) obtained after 1<sup>st</sup> flush, (E-F) obtained after 2<sup>nd</sup> flush taken at 250x magnification and 500x magnification



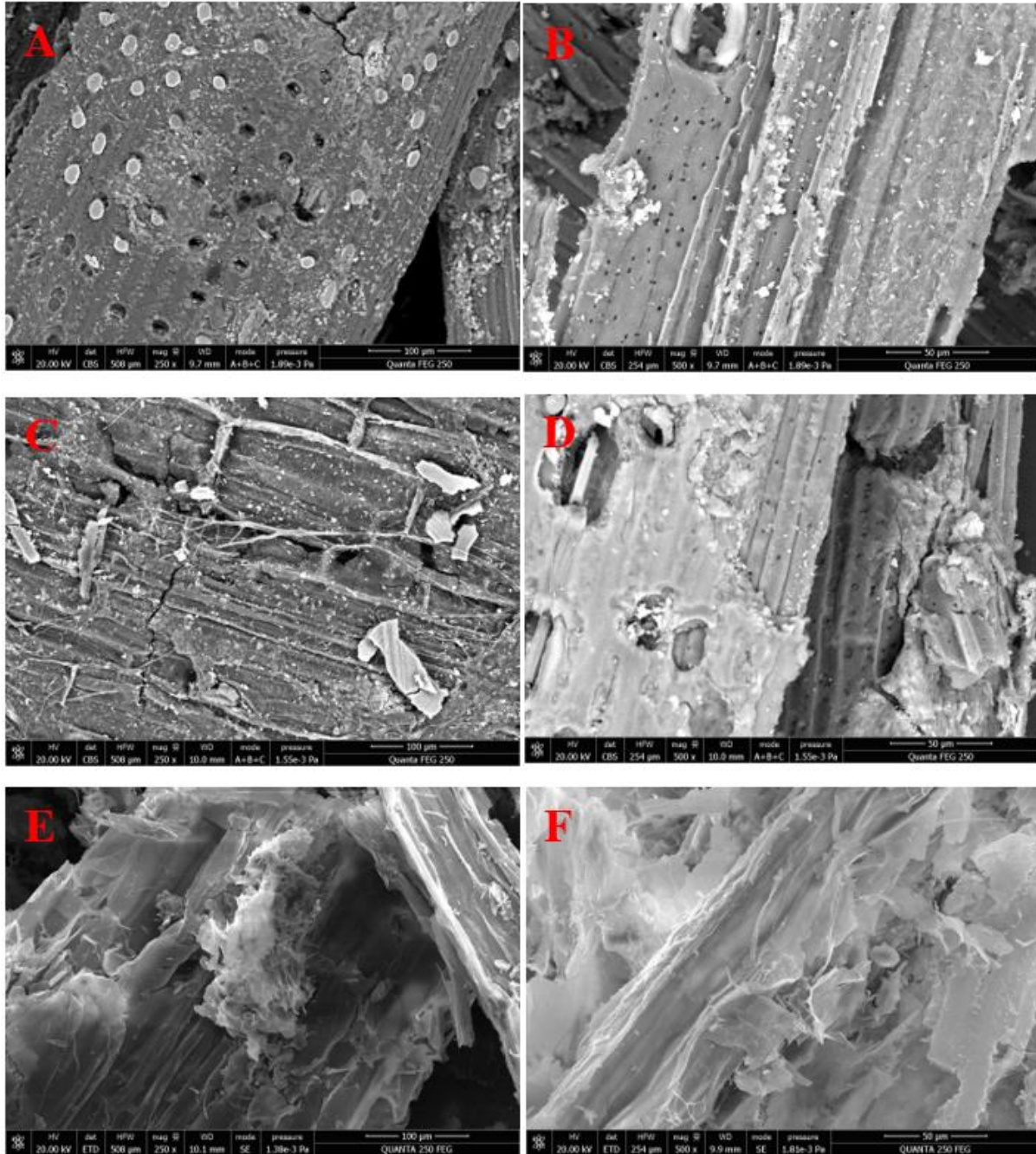


Figure V-6: SEM images of Spoppo strain SMWS (A-B) spawn sample, (C-D) obtained after 1<sup>st</sup> flush, (E-F) obtained after 2<sup>nd</sup> flush taken at 250x magnification and 500x magnification

### V.1.3.6 Biomethane Production

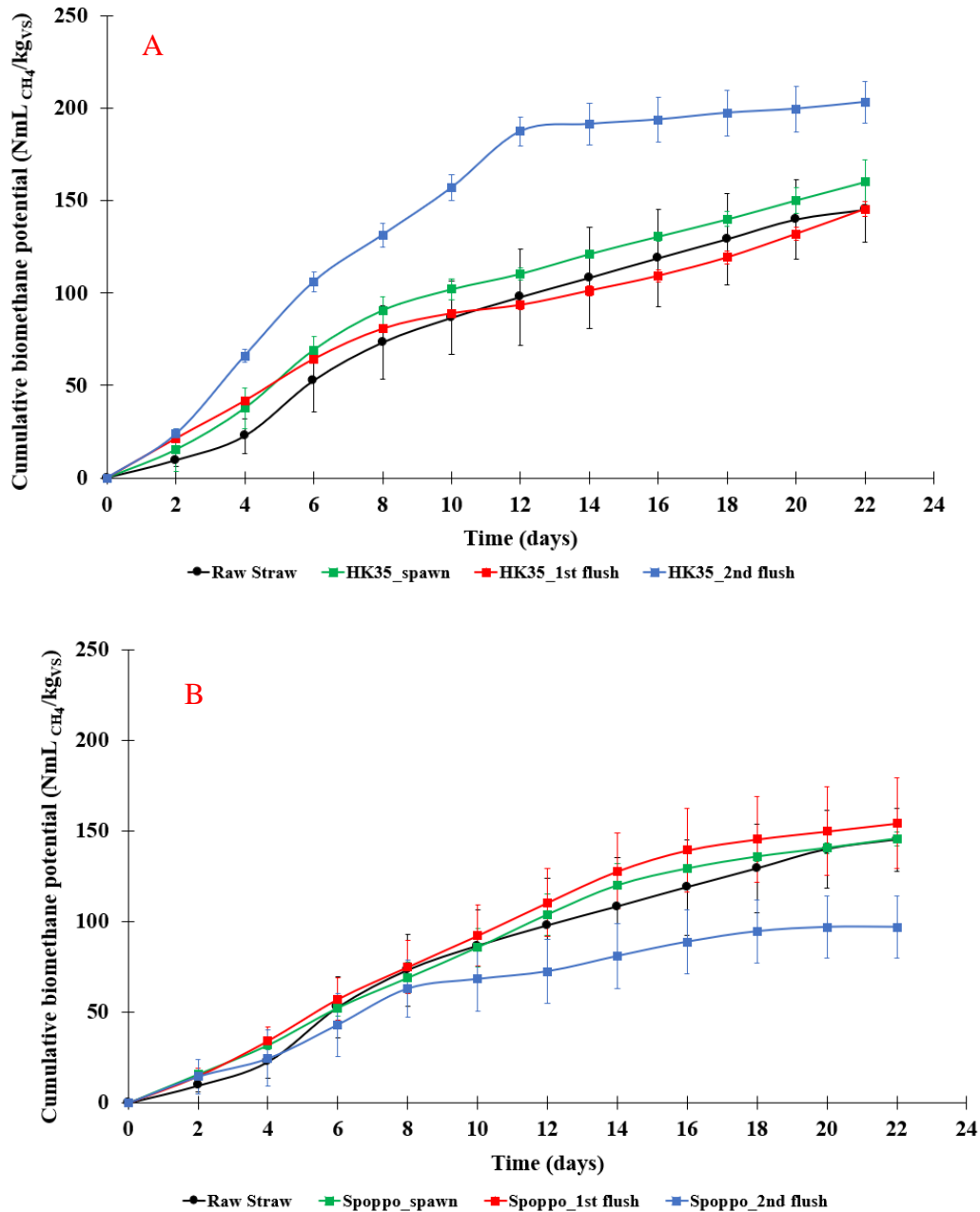


Figure V-7: Cumulative biomethane potential of raw straw and the SMWS samples of (A) HK35 and (B) Spoppo strain

Results are presented in terms of cumulative methane volume as a function of the time in Figure V-7. It can be observed that there was no significant difference in the BMP of raw straw and the SMWS substrates with spawn and after the first flush of both strains. For these samples, the BMP after 22 days of AD is in the range of 145 – 160 NmL<sub>CH<sub>4</sub></sub>/kg<sub>VS</sub>. This shows that though some poration was visible through SEM analysis, which could be caused due to dissolution of lignin and hemicellulose, it was not sufficient enough to facilitate subsequent hydrolysis in the AD process. There was a considerable difference in BMP after 22 days of the

2<sup>nd</sup> flush sample of HK35 ( $203.21 \pm 11.10 \text{ NmL}_{\text{CH}_4}/\text{kgvs}$ ) and Spoppo ( $96.90 \pm 17.15 \text{ NmL}_{\text{CH}_4}/\text{kgvs}$ ) strain. The structural modification as seen through SEM (Figure V-5 (E-F)), helped HK35\_2<sup>nd</sup> flush sample to have a faster hydrolysis compared to other samples. It is also worth recalling that HK35\_2<sup>nd</sup> flush had the lowest lignin content (23.5%). This confirms the potential of HK35\_2<sup>nd</sup> flush for facilitating the subsequent hydrolysis process and further increasing the biomethane production by 40% as compared to raw straw. Conversely, the Spoppo\_2<sup>nd</sup> flush sample had a higher lignin content (28.76%) than its counterparts and collapsed pores (SEM analysis). The decreased surface area for penetration of enzymes could have resulted in a low biomethane yield. The difference in the inherent characteristics of the two strains of *Pleurotus ostreatus* could have led to a difference in the fiber degradation capability that resulted in the variation in BMP. Similar results were observed in a study conducted by Nayan et al. (2018), where a variation was observed among strains within species in terms of growth patterns and fiber degradation capabilities. The contrary nature of the results obtained between the two strains could also be due to differences in the batch of wheat straw (variation in cultivars, maturity stages) used or the efficiency of strain to have invaded the entire batch of the substrate due to environmental conditions (temperature, humidity, oxygen levels) (Nayan et al., 2018). However, further genetic diversity studies and advanced techniques like Py/GC-MS are required to understand the difference in the capability of different fungal strains to modify and degrade the cell wall (Nayan et al., 2018).

#### **V.1.4 Conclusion**

The characterization of the spent mushroom substrate was carried out based on lignin composition, crystallite size, crystallinity index, and structural morphological changes using SEM and the results obtained were in concordance to understand the variation in the BMP. It showed that there is a variation among the fungal strains within the same species for their capacity to degrade the wheat straw for its growth. The HK35 was seen to utilize the easily available amorphous polysaccharides and then use lignin as a carbon source. Whereas, the Spoppo strain was seen to slowly use the amorphous polysaccharides which could be due to its slower growth rate. However, based on SEM and WAXD analysis, it can be assumed that they both have different biomass degradation methods. Spent mushroom wheat straw substrate after the 2<sup>nd</sup> flush of HK35 strain mushrooms had 22.5% lignin degradation which translated into a 40% increase in biomethane potential. Therefore, it was the best substrate for biogas production. For the mushroom industry, it is more resourceful to harvest 2 rounds of mushrooms using the same substrate and further to use the well-degraded substrate for integrated energy production. The outcome of this study underlines the importance to assess the different strains of the same

species and select the best strain for bioprocessing of the biomass. Therefore, three pure strains obtained from the mycology bank are compared for their biomass degradation ability in Chapter V.2. Nevertheless, the results obtained in Chapter V.1 offer knowledge to the mushroom industry to select the superior strain for their integrated biogas production.



## **V.2 Degradation of straw by three different strains of *Pleurotus ostreatus***

### **V.2.1 Introduction**

As seen in the previous sub-chapter V.1, the efficiency of fungal delignification varied among the strains of the same genus, however, there are various other parameters such as inoculum concentration, moisture content, aeration, pH, temperature, supplements, and incubation time which also affect the pretreatment effectiveness. To provide a more uniform environment and have comparable conditions, submerged fermentation was carried out. In many studies, screening fungal isolates for each substrate type has been shown to be important for optimizing the pretreatment process. A study was carried out by Nayan et al. (2017) to evaluate the differences between the two strains of *Ceriporiopsis subvermispora* for *in vitro* degradability of wheat straw. In this study by Nayan et al. (2017), clear variation between the strains was demonstrated based on their growth pattern and enzyme activities which led to differences in the degree of selectivity in lignin degradation. *Pleurotus ostreatus* has been seen to have a better degradation ability of various substrates as compared to *P. chrysosporium*, *T. versicolor*, or even *Pleurotus floridanus* (Nadir et al., 2019). Therefore, in this section, different strains of *Pleurotus ostreatus* obtained from the mycology bank are compared for their lignin degradation ability and increasing yield of fermentable carbohydrates in submerged conditions.

### **V.2.2 Materials and Methods**

#### **V.2.2.1 Biological materials**

Three strains of *Pleurotus ostreatus* (*Jacquin: Fries*) *Kummer*, namely, MUCL 20510, MUCL 28511, and MUCL 29420 were obtained from Belgium Coordinated Collections of Microorganisms (BCCM). These strains were selected because they were isolated from spawn on LCB. For easy naming of samples, MUCL 20510 would be referred to as PO1, MUCL 28511 as PO2, and MUCL 29420 as PO3 hereafter. Straw samples were obtained from Hamiform (in early 2019), a commercial brand that sells it as bedding for rodents and it mainly consists of wheat straw mixed with other straw. The straw was ground using a laboratory blender and sieved through a 2 mm mesh-size sieve and then again through 630  $\mu\text{m}$  to recover the retentate.

#### **V.2.2.2 Strain maintenance**

The strains were subcultured in MA1 agar medium (Malt extract (10 g) and Agar (15 g) in 1 L of demineralized water) every two months and incubated at 22°C for 3 weeks for the complete growth of mycelial mats in solid agar media. The Petri dishes were then stored at 4°C. 1 mL of a mixture of sterile demineralized water with 0.1% Tween 80 was used to loosen the

mycelia from the agar medium and viewed using an Olympus BX60 microscope attached to an Infinity 3 camera (Lumenera, Ottawa, ON, Canada).

#### V.2.2.2.1 Preculture preparation

2 mL of a mixture of sterile demineralized water with 0.1% Tween 80 was used to completely drench the mycelia on the agar medium. The absorbance of the mycelial solution was measured against the blank (demineralized water with 0.1% Tween 80). 1 mL of mycelial solution with an absorbance between 0.2 and 0.4 was added to 100 mL of sterile mineral media (MM) with a composition (based on the composition used in Velioglu and Ozturk Urek (2014)) as shown in Table V-3. A trace elements stock solution (Fayeulle, 2013) was prepared which consisted of 50 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O, 5 mg MnCl<sub>2</sub>·4H<sub>2</sub>O, 50 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 25 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 5 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 10 mg MoO<sub>3</sub> in 500 mL of demineralized water and autoclaved and stored separately at 4°C. The inoculated mineral media was left to incubate at 22°C for 2-3 weeks on an orbital shaker with day and night light variation.

**Table V-3:** Mineral media (MM) composition for fungal growth

Composition	g/ L
NH <sub>4</sub> NO <sub>3</sub>	0.724
KH <sub>2</sub> PO <sub>4</sub>	1
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1
KCl	0.5
Yeast Extract	0.5
Bacto-peptone	10
Glucose	10
Trace Elements stock solution	10 mL

#### V.2.2.2.2 Fungal pretreatment

Multiple flasks of preculture of each strain, namely, PO1, PO2, and PO3 were prepared and vacuum filtered under sterile conditions. The obtained mycelial mass for each strain was then homogenized with the help of sterile scalpels. Twelve flasks containing 5 g of straw and 100 mL mineral media (Table V-3) without glucose were autoclaved. Three flasks for each strain were then incubated with 1 g fresh weight of the homogenized mycelia. Control for this experiment was also prepared in triplicates with 5 g of straw and 100 mL MM without glucose and any inoculation. All the flasks were left to incubate in a shaker incubator at 22°C for 30 days. The control and fungal pretreated samples were then vacuum filtered in sterile conditions. The obtained straw was then dried at 40°C for 3 – 4 days. The dried straw was then used for further analysis.

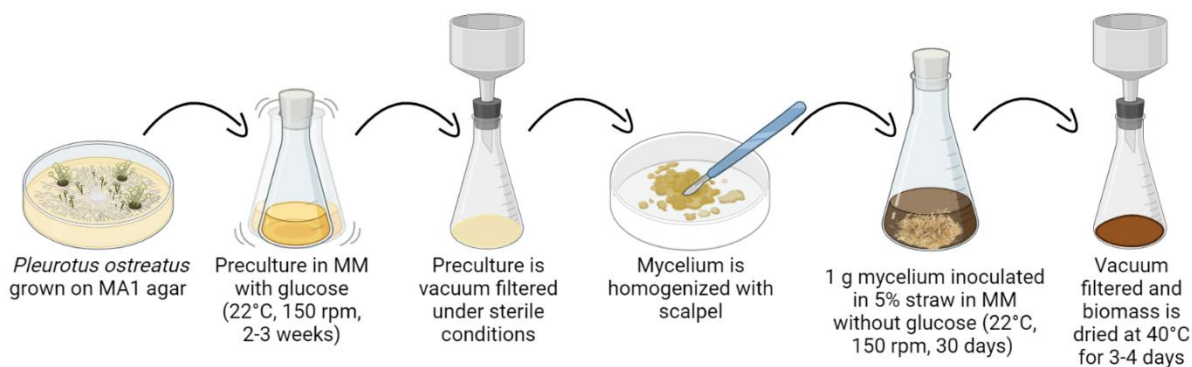


Figure V-8: Schematic representation of fungal pretreatment of straw. Created with BioRender.com

### V.2.2.2.3 Characterization of straw

TS, VS, and elemental composition were determined according to the protocol described in IV.2.2.5.1. The surface morphology was studied using SEM as described in IV.2.2.7.1. Lignin composition was determined according to the NREL method (Sluiter et al., 2012). The hydrolysate obtained during lignin compositional analysis was used to determine the sugars using High-Performance Liquid Chromatography (HPLC) (Shimadzu, France). The hydrolysate was neutralized with  $\text{CaCO}_3$  and filtered using a 0.2  $\mu\text{m}$  syringe filter and used for the analysis. The HPLC instrument was equipped with a Biorad Aminex HPX-87P column and an ELSD detector. The analysis was carried out at 80°C with a flow rate of 0.6 mL/minute and degassed demineralized water for the mobile phase. Standards were run for glucose and xylose at different concentrations to plot a calibration curve based on which the glucose and xylose concentrations in the samples were estimated. All the tests were carried out in triplicates.



## V.2.3 Results and Discussion

### V.2.3.1 Strains Morphology

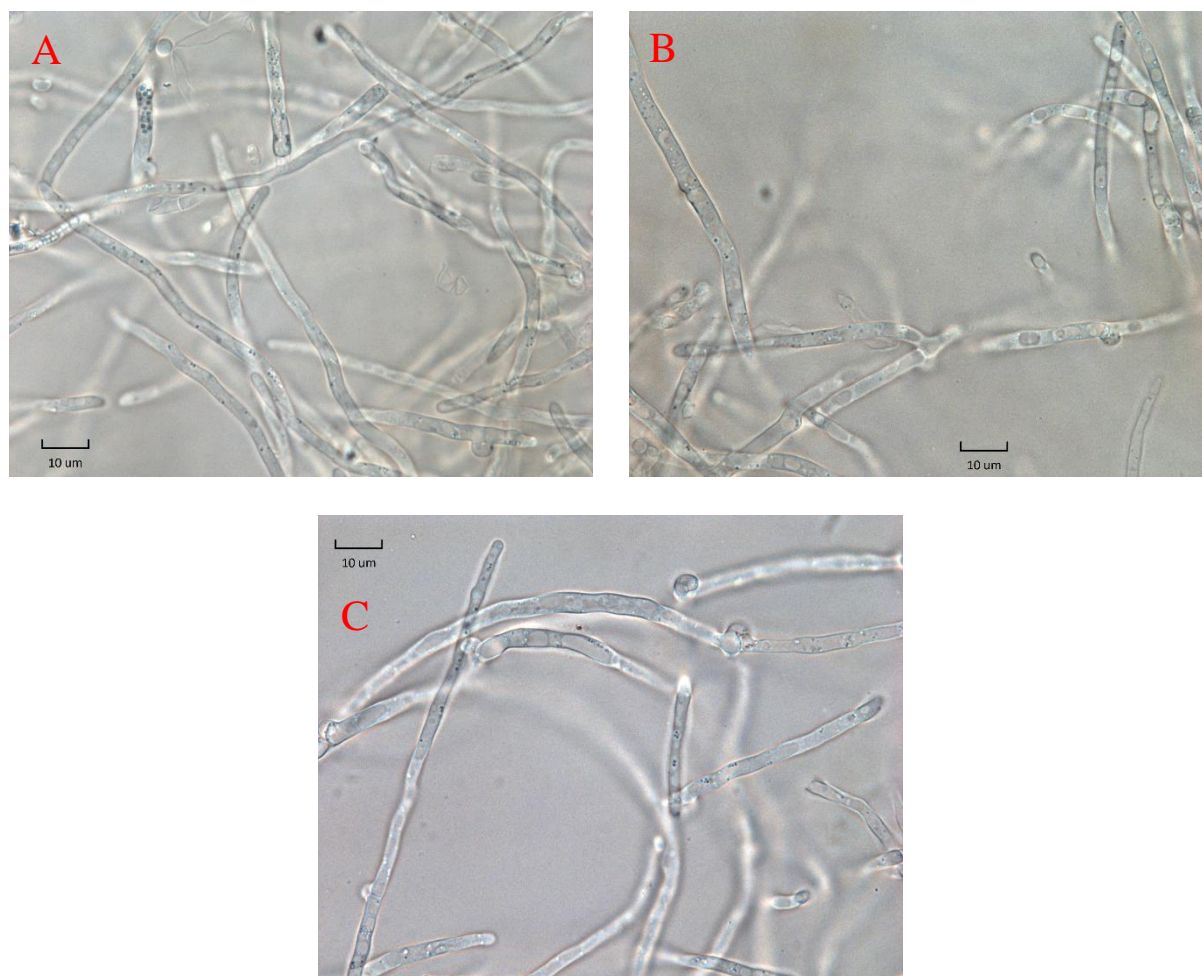


Figure V-9: Strain morphology of (A) PO1, (B) PO2, (C) PO3 as observed using 100x magnification.

Typical hyphal features such as cell walls, cross walls or septa, and vacuoles are seen in all strains (Figure V-9 (A-C)). No difference is observed between the strains in terms of hyphal structure. The morphological features such as mycelium color on agar plates or in preculture medium also did not vary. However, the growth rate of mycelium on solid agar was seen to be similar for PO3 and PO2 while it was slower in PO1.

### V.2.3.2 TS, VS, and Elemental Composition

The TS and VS in all the samples studied here were  $97.95 \pm 0.17$  % and  $96.90 \pm 1.01$  % respectively. The elemental composition between the control and fungal pretreated samples also did not vary significantly. The percentages of nitrogen, carbon, hydrogen, sulfur, and oxygen were  $0.8 \pm 0.15$ ,  $43.4 \pm 0.4$ ,  $5.6 \pm 0.02$ ,  $0.05 \pm 0.01$ , and  $35.0 \pm 0.5$  respectively.

### V.2.3.3 Lignin Composition

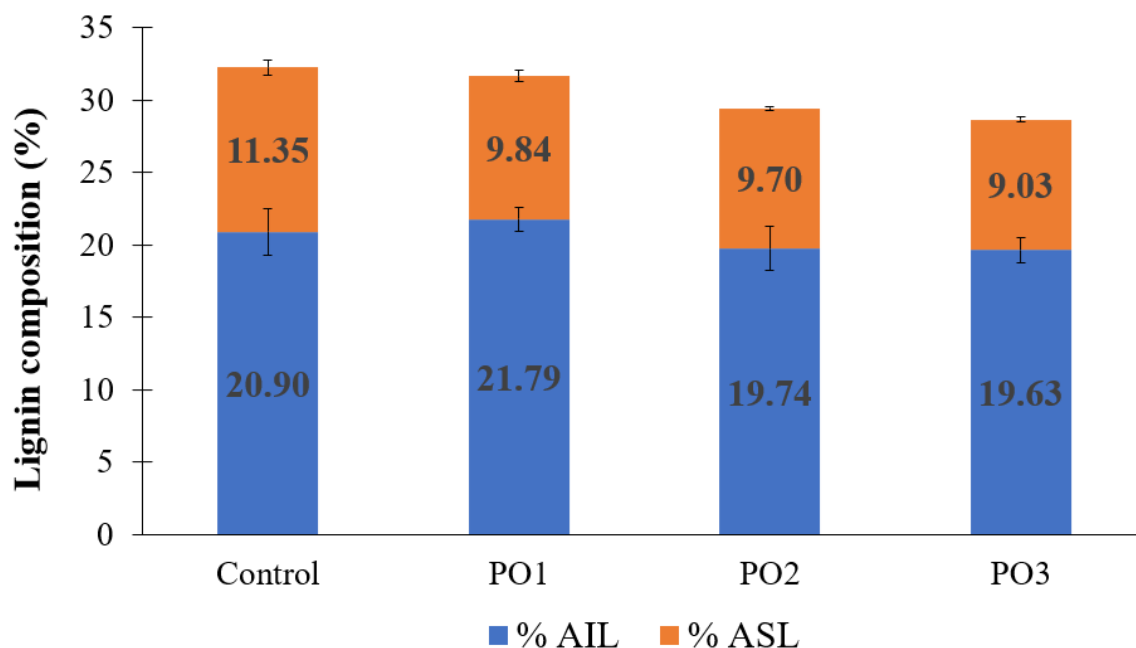


Figure V-10: Lignin composition of control and fungi pretreated straw

A particular feature of white-rot fungi is that lignin degradation does not occur during fungal growth but only after nutrient depletion which triggers its secondary metabolism. The lignin degradation is too slow to serve as a source of energy for the fungi. Therefore, WRF can degrade lignin only in the presence of carbohydrates such as cellulose or hemicellulose which act as an alternative energy source (Castoldi et al., 2014). From Figure V-10, we can observe that there is a variation in the lignin degradation efficiency of the different strains within the same species. PO1 had the lowest lignin degradation (2%) while PO3 showed the highest lignin degradation (11%). This could be due to PO1 having a slower growth rate compared to PO2 and PO3. A similar difference was observed in a study where 20 strains of *P. eryngii* was compared for their ability to degrade wheat straw which was associated with differences in geographical location and origin (wild or cultivated) of the strain where it was isolated (Sonnenberg et al., 2016).

### V.2.3.4 Structural Carbohydrates

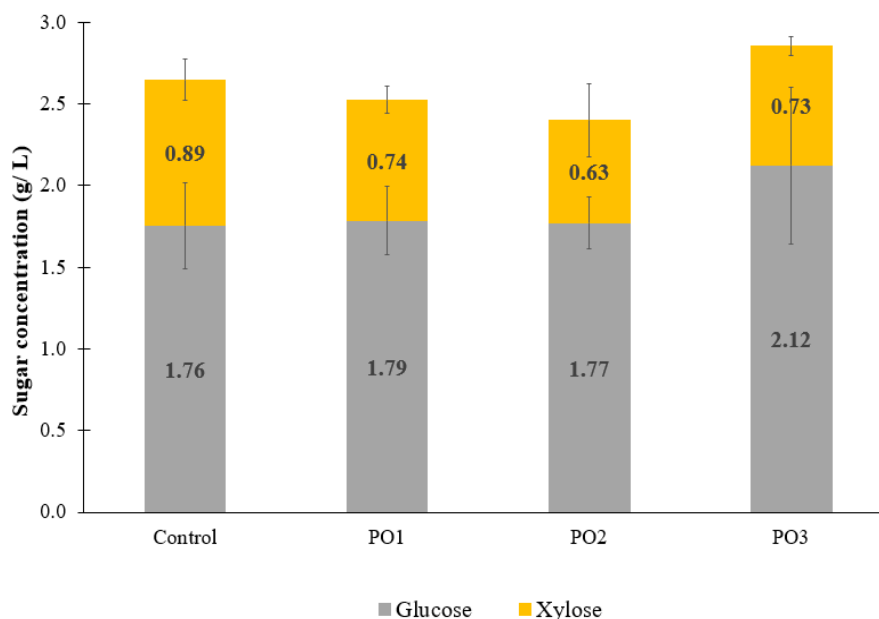


Figure V-11: Sugar monomers concentration (g/L) of fungi pretreated straw

The prevention of loss of carbohydrates in the pretreated biomass is an important parameter than lignin degradation for improved biogas production. Therefore, the detection of sugar monomers – glucose and xylose were carried out using HPLC. The PO3 pretreated biomass exhibited a slightly higher sugar concentration of 7.5% (Figure V-11) after acid hydrolysis of the biomass amongst the strains compared. Therefore, the PO3 strain seems to be slightly better than the PO2 and PO1 strains in terms of avoidance of loss of carbohydrates.

### V.2.3.5 Structural Morphology of Straw

Scanning electron microscopy helped to visualize the rigid and highly ordered fibrils in the control sample (Figure V-12 (A-B)). After the fungal pretreatment for 30 days, visible changes in the size of the straw particles were observed after vacuum filtration.

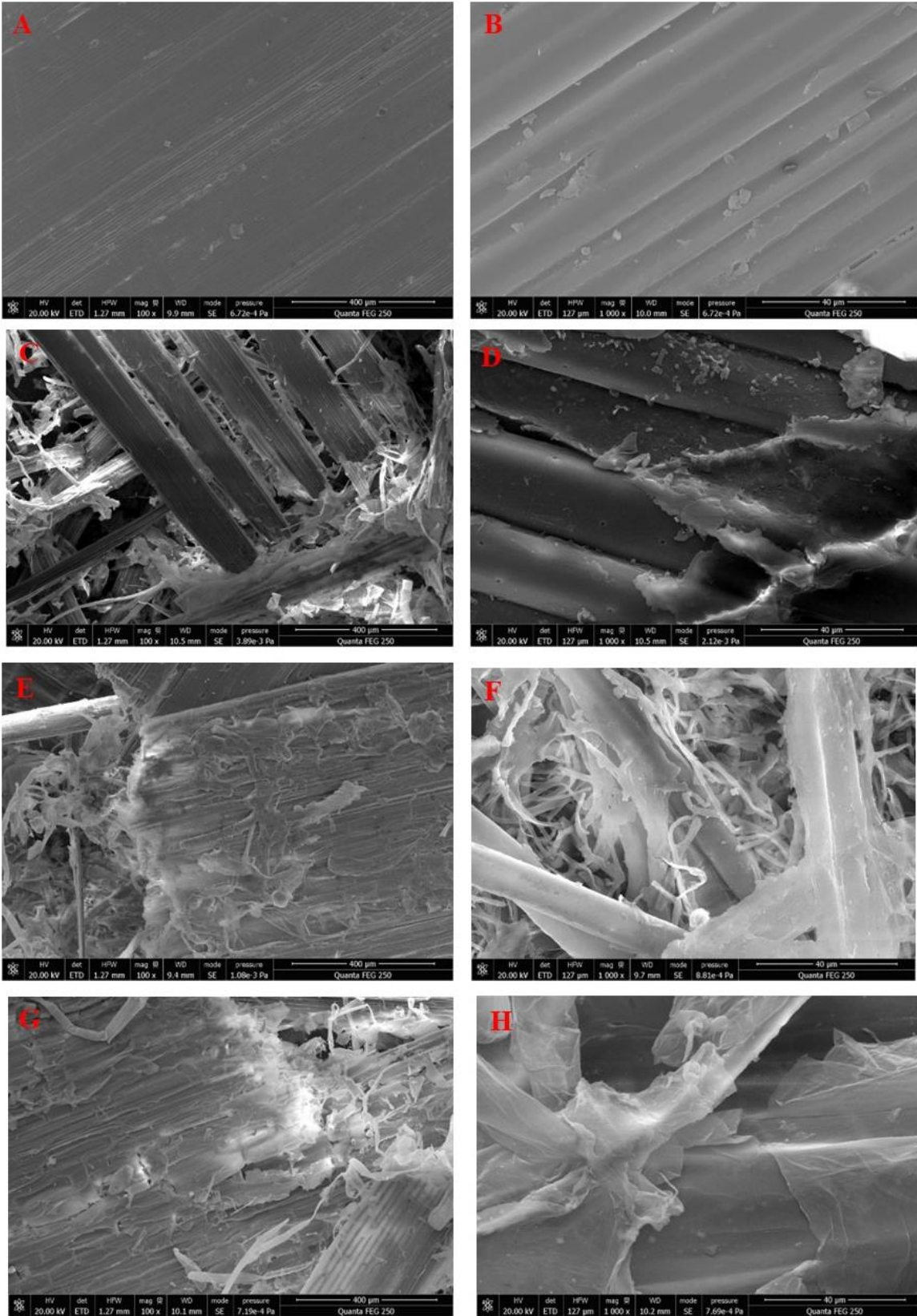


Figure V-12: SEM images taken at 100x and 1000x magnification of (A-B) control, (C-D) PO1 pretreated, (E-F) PO2 pretreated, and (G-H) PO3 pretreated straw

However, the size reduction was not uniform throughout all the straw particles. Therefore, the variation was observed in the SEM scans as well. There was no appearance of pores in any of these strains of fungi-pretreated biomass (Figure V-12 (C-H)). However,

disorganization of the straw fibers is visible in all the fungi pretreated samples. Similar results were obtained after the treatment of Eucalyptus sawdust with *P.ostreatus* where substantial modification in fibers was observed without the formation of pores (Castoldi et al., 2014).

#### **V.2.4 Conclusion**

To have an efficient pretreatment process, selective delignification along with low loss of high fermentable sugars is needed. The PO3 strain showed an 11% reduction in lignin content and a 7.5% increase in sugar production. However, a variation in the efficiency of fiber degradation and carbohydrate production was observed among the three strains of *Pleurotus ostreatus* obtained from a mycology bank. This indicates the possibility of genetic variation and enzyme activity variation between the strains. In order to select the right isolates for the pretreatment process, screening a large number of fungal isolates is important. Genomic studies would help breeders to develop strains to improve the utilization of biomass and thus yields of biogas production.

### **V.3 A perspective summary of Chapter V**

Straw is very low in nitrogen content and is a good growth substrate for mushrooms, however, fungi-depleted straw is better for biogas production since the C/N ratio is reduced and is closer to the optimum for AD process. However, evident differences in degradation ability among the strains of the same genus were observed in both submerged and solid-state cultivations. Even though solid-state cultivations of fungi were able to produce up to a 40% increase in biogas production, it varied among the strains due to the extent of utilization of carbohydrates for its growth. However, in submerged conditions, the variations in the degradation ability among the strains were more subtle. Therefore, submerged conditions were used for further studies. Based on the results obtained in this chapter, the best strains among the hybrid and pure types, namely HK35 and PO3, were chosen for further studies. With the selected conditions, the next chapter explores the combined fungal and chemical pretreatment of straw for biogas production.

# VI Combined fungal and chemical pretreatment of lignocellulosic biomass

## VI.1 Introduction

As fungi grow, they generate different acidic groups like phenolic acids and other simple organic acids. These acidic compounds reduce the pH of the environment and help to solubilize iron and make them available for the Fenton reaction. Consequently, white rot fungi (WRF) apply their own Fenton-like reaction to degrade the biomass. Therefore, a combination of fungal and oxidative pretreatment processes could initiate a synergistic effect which could help to improve the yields of the desired end product (Hashemi et al., 2022). In combined pretreatment strategies, the order of sequential pretreatment steps may also influence the relative components of the biomass and therefore, the biofuel yield (Meenakshisundaram et al., 2021). Therefore, fungal followed by chemical pretreatment and the reverse order, chemical followed by fungal pretreatment was studied against sole fungal pretreatment. Based on the previous screening study for fungal strains in Chapter V and based on chemical pretreatment studies in Chapter IV, fungal pretreatment with HK35 and PO3 strains and low concentration Fenton reaction were carried out without much size reduction and autoclaving process to lower the energy needs of the pretreatment process. The synergy of oxidative-fungal pretreatment on the modifications of the chemical composition of the biomass, crystallinity, morphology and biomethane potential were all investigated.

## VI.2 Materials and Methods

### VI.2.1 Materials

All chemicals of reagent grade were obtained from VWR, Rosny-sous-Bois Cedex, France, and used without further purification. Demineralized water (18 M $\Omega$ ·cm) was used to make all the solutions. Straw samples were obtained from Hamiform (in December 2021). Straw was ground using a laboratory blender (Waring commercial) and sieved first through 4 mm and then through a 2.5 mm sieve to recover the retentate (2.5 mm – 4mm).

*Pleurotus ostreatus* (Jacquin: Fries) Kummer MUCL 29420 (PO3) was obtained from Belgium Coordinated Collections of Microorganisms (BCCM). *Pleurotus ostreatus* HK35 strain was isolated and used according to section V.1.2.2.1. For both strains, the pre-culture was prepared according to the protocol mentioned in section V.2.2.2.1. Multiple flasks of preculture of each strain, PO3 and HK35 were prepared and vacuum filtered under sterile condition. The obtained mycelial mass for each strain was then homogenized with the help of sterile scalpels.

## **VI.2.2 Pretreatment Strategies**

### **VI.2.2.1 Sole Fungal Pretreatment**

Six flasks containing 15 g of straw were added with 300 mL of autoclaved mineral media (Table V-3) without glucose. Three flasks for each strain were then incubated with 1 g of the homogenized mycelia. Control for this experiment was also prepared in triplicates with 15 g of straw added with 300 mL of autoclaved MM without glucose and any inoculation. All the flasks were left to incubate in a shaker-incubator at room temperature for 30 days. The control and fungal pretreated samples were then washed (with sterile demineralized water) and vacuum filtered under sterile conditions. The obtained straw was then dried at 40°C for 3 – 4 days. The dried straw was then used for further analysis. These samples are hereafter referred to as PO3\_only FP and HK35\_only FP.

### **VI.2.2.2 Fungal followed by chemical pretreatment (FP+CP)**

Similar to the protocol followed in section VI.2.2.1, 6 flasks were prepared (triplicates for each strain). After 30 days of incubation, the obtained straw was vacuum filtered and dried. The weight of the straw was measured for each flask and added with sterile demineralized water to obtain 5% substrate consistency. The pH was reduced to 3 using dilute sulphuric acid (1 M). Chemical pretreatment was carried out with 1.065 mM Fe<sup>2+</sup> and 0.15 M H<sub>2</sub>O<sub>2</sub> for every 100 mL of demineralized water added. The flasks were then let to incubate at room temperature for 24 hours in a shaker-incubator. The straw was then washed and vacuum filtered and dried at 40°C for 3-4 days. These straw samples are hereafter labeled as PO3\_FP+CP and HK35\_FP+CP.

### **VI.2.2.3 Chemical followed by fungal pretreatment (CP+FP)**

Six flasks with 15 g of straw each were added with 284 mL of demineralized water and the pH was reduced to 3 with the help of dilute sulphuric acid (1 M). Chemical pretreatment was carried out first with 190.65 mg of FeCl<sub>2</sub>·4H<sub>2</sub>O (1.065 mM Fe<sup>2+</sup>) and 15.3 mL of 30% H<sub>2</sub>O<sub>2</sub> (0.15 M) for all the flasks. The flasks were then incubated at room temperature for 24 hours in a shaker-incubator. The straw was then washed, vacuum filtered, and dried at 40°C for 3-4 days. The straw was then added with 300 mL autoclaved MM without glucose. Three flasks for each strain were then incubated with 1 g of the homogenized mycelia. All the flasks were left to incubate in a shaker-incubator at room temperature for 30 days. The obtained straw was then dried at 40°C for 3 – 4 days. Henceforward, these straw samples are referred to as PO3\_CP+FP and HK35\_CP+FP.

### VI.2.3 Biomass Characterization

The biomass was characterized for TS, VS, and lignin composition as shown in section IV.2.2.5.1 and section IV.2.2.5.3 respectively. The surface morphology of the biomass was observed using SEM as described in section IV.2.2.7.1. The crystallinity was studied using X-ray diffraction as mentioned in section IV.2.2.7.4.

### VI.2.4 Biomethane Potential Test

Two AMPTS systems were set up as described in section IV.2.2.6 to estimate the biomethane potential of control, PO3\_only FP, PO3\_FP+CP, PO3\_CP+FP, HK35\_only FP, HK35\_FP+CP, HK35\_CP+FP in triplicates. The AMPTS system at Institut Polytechnique UniLaSalle, Beauvais was used for studying control and HK35 samples while the AMPTS at UTC was used for studying PO3 samples. The two systems were started on the same day with the same inoculum from a large-scale anaerobic digester digesting agricultural residues in Coudon, France. Before the start of the experiment, the inoculum was left to stabilize for a week at room temperature and then filtered through a 1 mm porosity sieve to remove unwanted particles. The inoculum had a TS and VS of  $7.24 \pm 0.02$  % and  $64.06 \pm 0.08$  % respectively and a FOS/TAC ratio of  $0.199 \pm 0.003$ .

## VI.3 Results and Discussion

### VI.3.1 Biomass Characterization

The %TS and VS (%TS) of control and pretreated straw are represented in Table VI-1.

**Table VI-1:** TS and VS of control and pretreated straw

Sample	TS (%)	VS (%TS)
Control	$90.22 \pm 2.47$	$98.57 \pm 0.32$
PO3_only FP	$91.44 \pm 0.26$	$96.64 \pm 0.25$
PO3_FP+CP	$91.32 \pm 0.17$	$95.87 \pm 4.99$
PO3_CP+FP	$92.02 \pm 0.49$	$98.74 \pm 0.16$
HK35_only FP	$98.46 \pm 0.35$	$97.01 \pm 0.26$
HK35_FP+CP	$91.42 \pm 4.12$	$95.42 \pm 5.56$
HK35_CP+FP	$94.12 \pm 0.06$	$98.06 \pm 0.35$



### VI.3.2 Lignin Composition

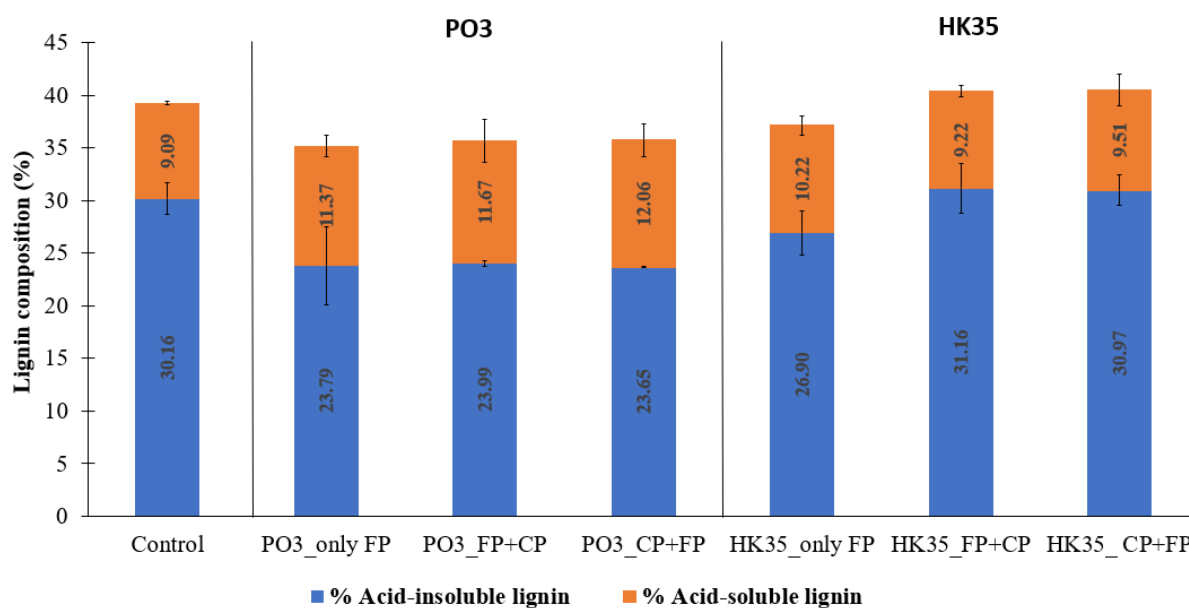


Figure VI-1: Lignin composition in terms of %ASL and %AIL of sole fungal pretreated and combined pretreatment

From Figure VI-1 it can be observed that PO3 samples under all conditions showed an overall lignin degradation of  $9.5 \pm 0.6$  % compared to the control. A similar result of 11% of lignin degradation was obtained by PO3 studied in similar conditions in chapter V.2.3.3. Subsequent chemical pretreatment does not improve lignin degradation by PO3 nor PO3 is disturbed by the chemical pretreatment when it occurs first. Sole HK35 pretreatment degrades less lignin (5%) as compared to control under the same conditions as PO3. The combined pretreatment with HK35 and Fenton reaction (in any order of pretreatment) did not cause any lignin content change as compared to the control.

### VI.3.3 Crystallinity index and crystallite size using WAXD

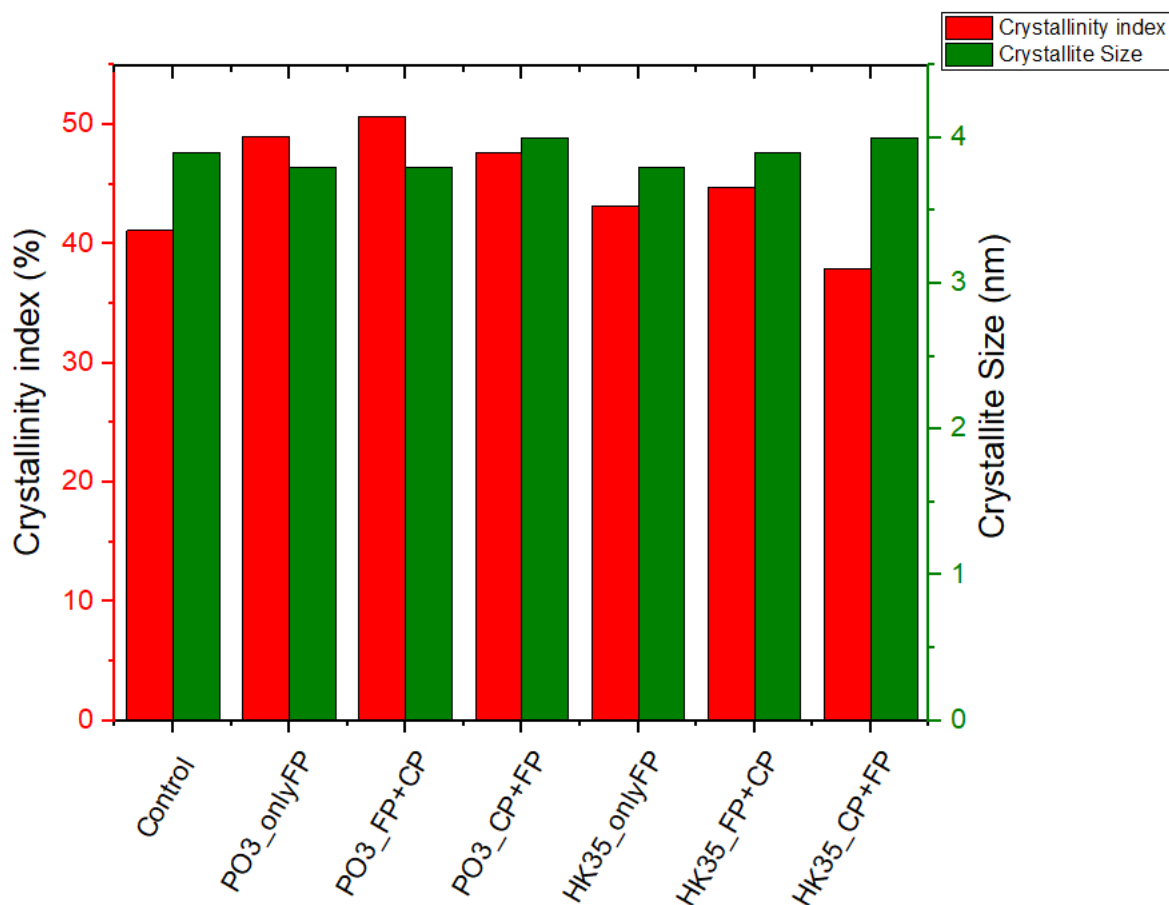


Figure VI-2: Crystallinity index and crystallite size of sole fungal and combined pretreated biomass

CI measurement of cellulose by XRD provides a qualitative or semi-quantitative evaluation of the amounts of amorphous and crystalline cellulose components (Karimi and Taherzadeh, 2016). From Figure VI-2, it can be observed that the crystallinity index of the PO3 pretreated sample is 49% as compared to 41% in the control. The increase in CI is a result of the removal of amorphous components in the biomass, mostly hemicellulose and cellulose. Thus, PO3 degrades amorphous polysaccharides and lignin (Figure VI-1 and Figure VI-2). The crystallinity index among the pretreated samples had a similar trend among the two strains. In general, the crystallinity index was FP+CP > only FP > CP+FP. This shows that the fungal pretreatment when carried out first, degrades the amorphous region. Subsequent chemical pretreatment seems to further degrade the amorphous cellulose which increases its crystallinity index. On the other hand, chemical pretreatment when carried out first makes the less available cellulose accessible to fungal degradation and the crystallite size seems to increase to 4.0 nm in this case. The HK35 only degrades a little the available cellulose and therefore there is only a slight increase in the crystallinity index in HK35\_only FP and HK35\_FP+CP samples. The chemical pretreatment first attacks the crystalline cellulose, which provides the HK35 with a

more amorphous region to degrade. This result is in contrast to the results of HK35 obtained in chapter V.1.3.4. This is because, in chapter V.1.3.4, the growth of the mushroom took place in solid state conditions whereas in this chapter, the study was conducted in submerged conditions. Moreover, the incubation time of HK35 strain on the substrate is different in both studies.

### VI.3.4 Scanning Electron Microscopy Analysis

The surface of the control sample (Figure VI-3) seems to be slightly modified as it was soaked in mineral media for 30 days. The main difference that can be seen from SEM analysis between the two strains is that the HK35 strain (Figure VI-5) has a mode of action that predominantly makes the biomass porous whereas the PO3 (Figure VI-4) strain does not. This difference can also be observed in sections V.1.2.3.1 and section V.2.3.5. Therefore, it is safe to assume that HK35 prefers to degrade the carbohydrates more than lignin as opposed to PO3. Figure VI-4 (A1) and (A2) show the lateral and cross-section of straw respectively. The transversal section shows that the fungus would be able to colonize the straw both superficially and intracellularly. The macropores generated as generally due to the dissolution of lignin and/or hemicellulose/ cellulose content are observed in HK35 pretreated biomass (Figure VI-5 (A1 and A2)).

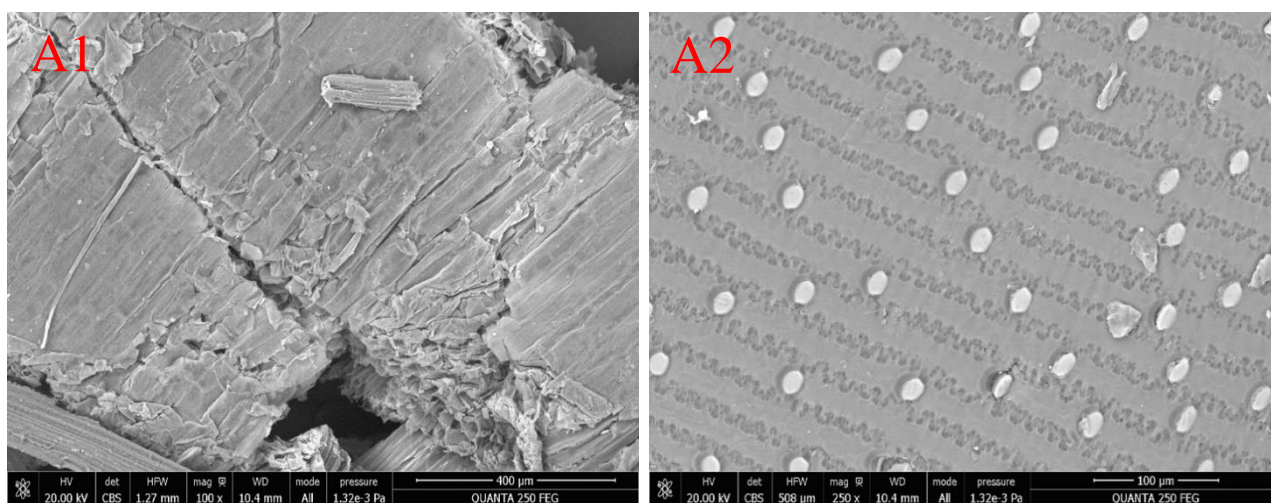


Figure VI-3: SEM image of (A) control sample taken at (1) 100x magnification and (2) 250x magnification

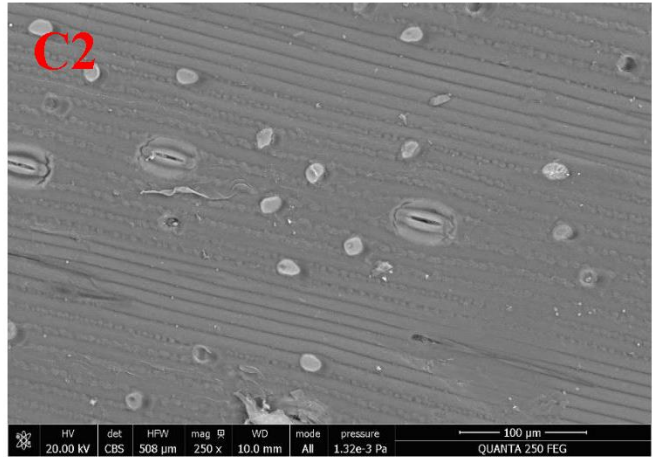
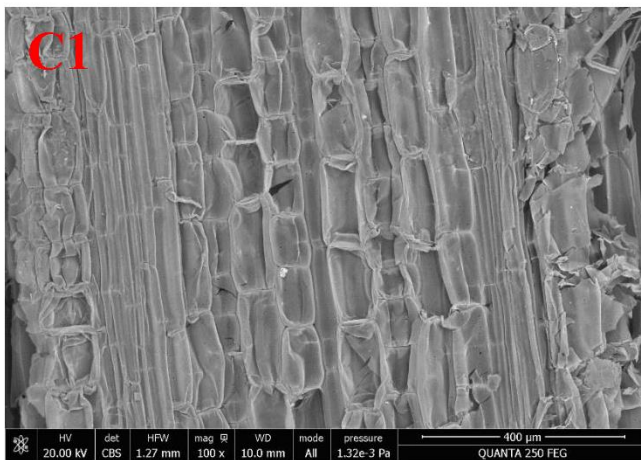
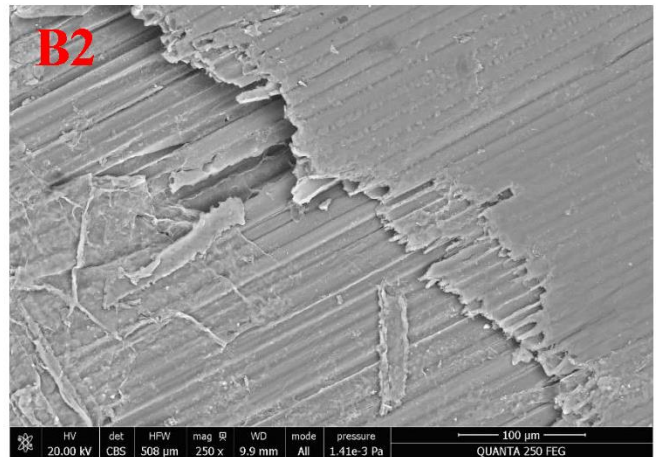
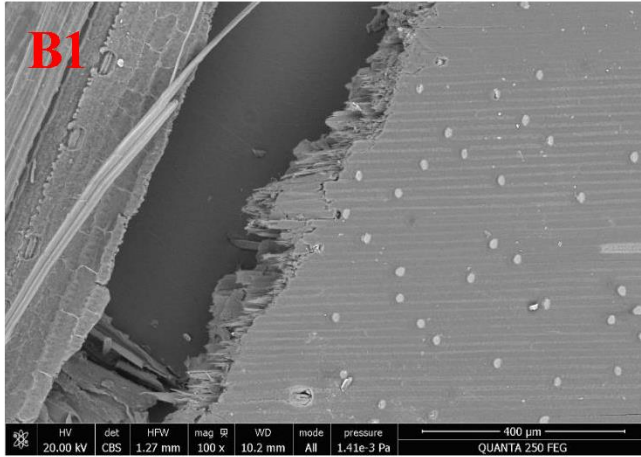
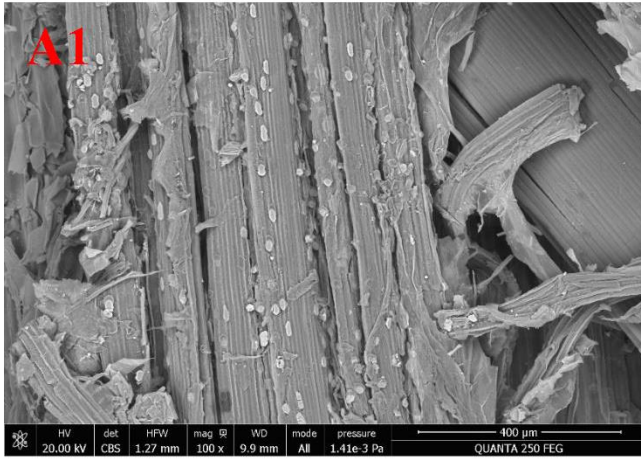


Figure VI-4: SEM image of (A) PO3\_only FP sample, (B) PO3\_FP+CP, (C) PO3\_CP+FP taken at (1) 100x magnification and (2) 250x magnification

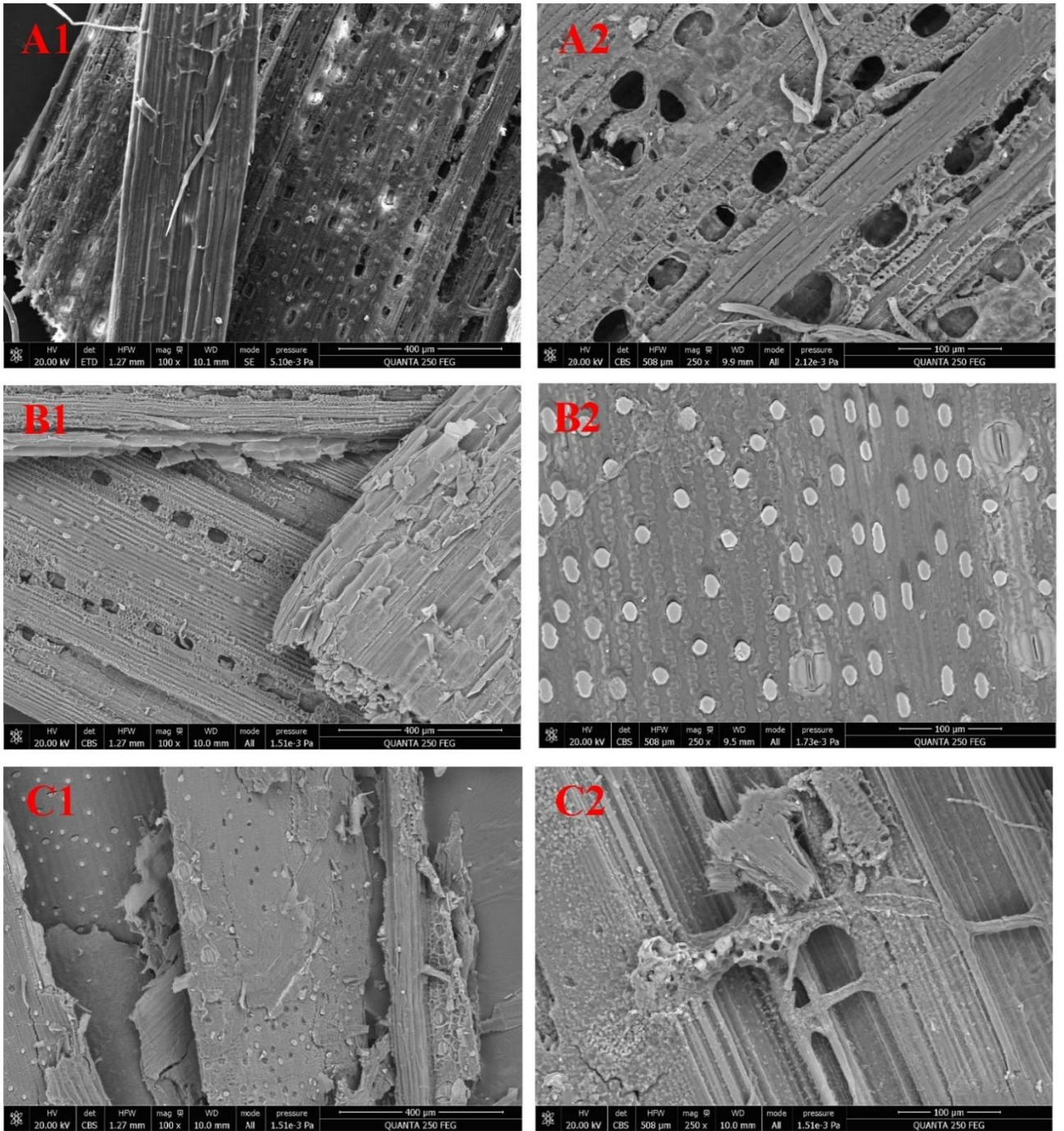


Figure VI-5: SEM image of (A) HK35\_only FP sample, (B) HK35\_FP+CP, (C) HK35\_CP+FP taken at (1) 100x magnification and (2) 250x magnification

### VI.3.5 Biomethane Potential Test

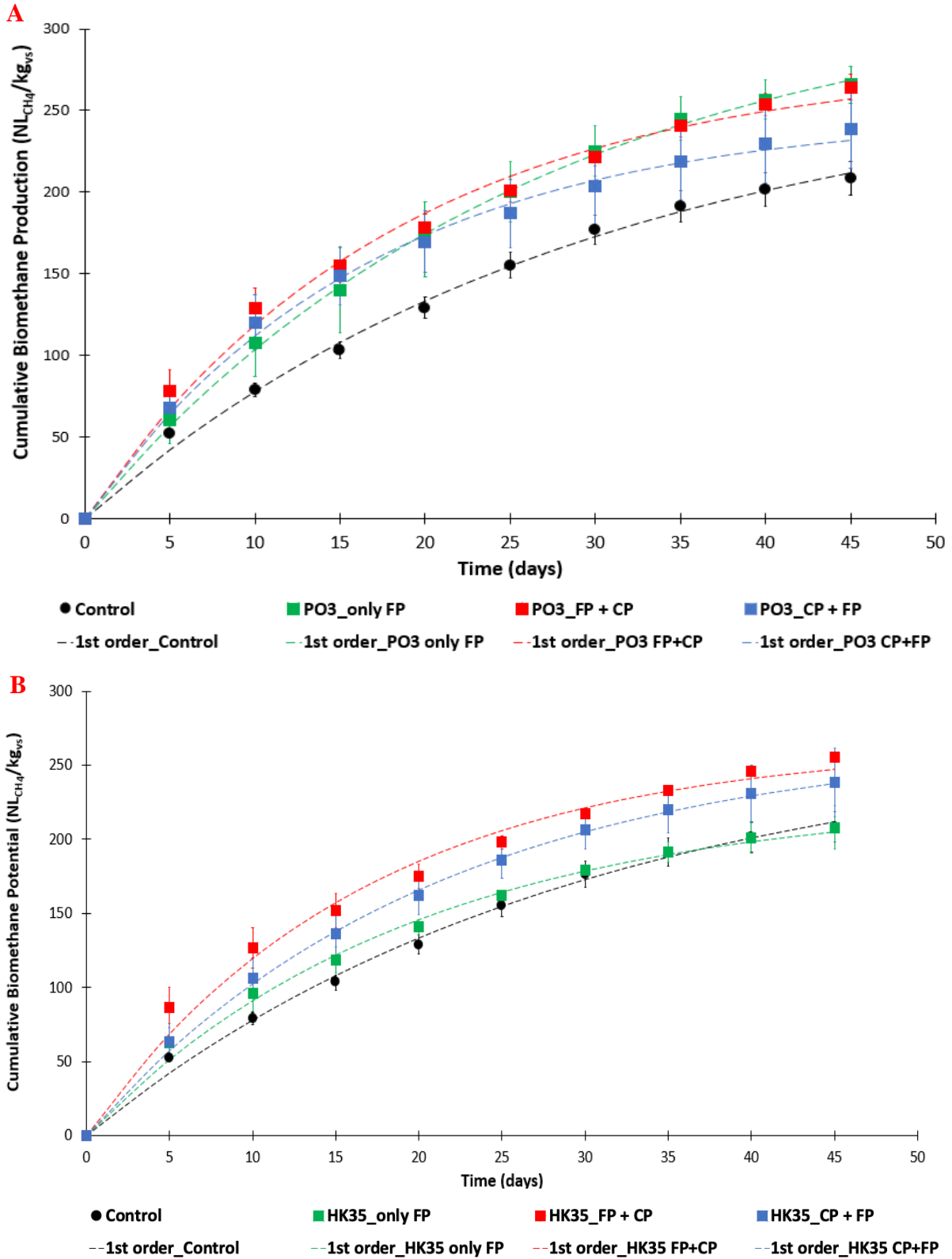


Figure VI-6: Cumulative biomethane potential over time for (A) PO3 and (B) HK35 samples

From Figure VI-6, pretreatment with the PO3 fungi alone (PO3\_only FP) is observed to increase the BMP by 27.5% as compared to the control. PO3 showed a comparatively higher lignin reduction which helped to improve the BMP. Subsequent treatment with oxidants

(PO<sub>3</sub>\_FP+CP), does not radically benefit the effect of fungal pretreatment with PO<sub>3</sub>. In the case of HK35\_only FP, there was no lignin degradation observed nor an increase in the biofuel yield as compared to the control. However, the combined pretreatment in the case of HK35 helped to increase the biomethane potential. In correlation with the crystallinity analysis (section VI.3.3), when chemical pretreatment (CP+FP) is carried out first, more of the crystalline cellulose is made available for fungal consumption and therefore, lowers the biofuel yield in the case of both PO<sub>3</sub> and HK35. This can be observed in Figure VI-7 (C) as formation of fruiting body. In comparison with oyster mushroom conditions studied in chapter V.1.3.6, the cultivation time, temperature, inoculum, etc. are quite different, and therefore, the BMP obtained after pretreatment by HK35 in submerged (chapter VI.3.5) and solid-state cultivation (chapter V.1.3.6) is different. Solid-state culture after 2 flushes with HK35 produced 22.5% lignin degradation and a 40% increase in BMP as compared to raw straw (chapter V.1.3.6). Nevertheless, the lignin degradation through fungal solid-state culture could produce phenolic compounds that could affect the digestibility negatively and also pose problems in the scale-up process. Submerged cultivation has the advantage of the uniform availability of nutrients throughout, control of agitation, pH, etc. which makes handling in bioreactors for large-scale cultivation easier.

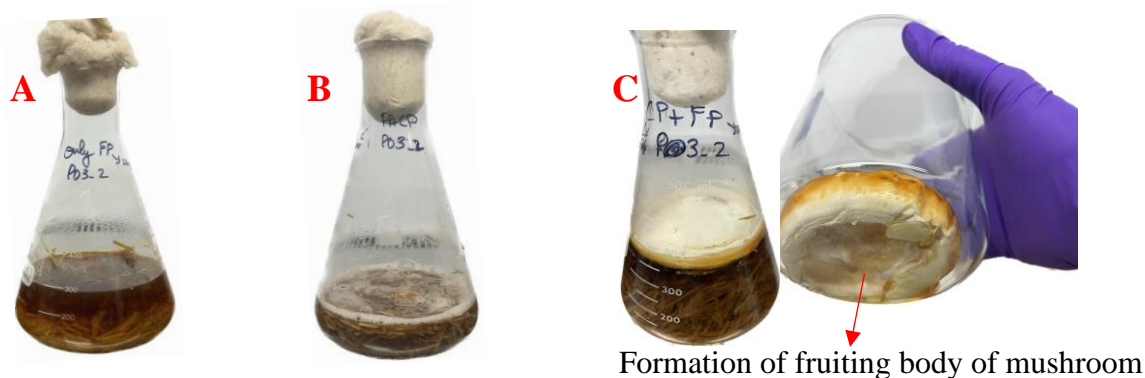


Figure VI-7: Pretreated samples after 30 days (A) PO<sub>3</sub>\_only FP, (B) PO<sub>3</sub>\_FP+CP, and (C) PO<sub>3</sub>\_CP+FP

However, the main problem of conducting fungal pretreatment in submerged conditions is the formation of complex mycelial clumps. The fungi mycelium at the center seem to clump the straw and form small pellets. Figure VI-8 shows an example of the vacuum-filtered sample after pretreatment with PO<sub>3</sub> for 30 days in submerged conditions. This results in visible particle size change due to fungal degradation. However, this change is not homogenous. This prevents efficient degradation of biomass throughout submerged conditions.



Figure VI-8: PO3 fungal pretreated biomass after submerged cultivation for 30 days

#### VI.4 Conclusion and a perspective summary of Chapter VI

Two strains of *Pleurotus ostreatus*, namely, HK35 and MUCL 29420 (PO3) were used to pretreat straw in submerged cultivation. The effect of fungal-oxidative combined pretreatment on straw was also studied for biogas production. Differences were observed in the biomethane yield of the same substrate pretreated with the two strains of the same species. *Pleurotus ostreatus* MUCL 29420 in submerged cultivation was observed to degrade lignin and enhance biomethane yield by about 27.5%. Whereas the *Pleurotus ostreatus* HK35 strain cultivated for 30 days in submerged conditions did not improve the biomethane yield of straw. However, the addition of low-concentration oxidants pretreatment to HK35 pretreated biomass did considerably improve the biomethane production while it was not the case for PO3 strain. For both strains, chemical pretreatment followed by fungal pretreatment proved to be inhibitory to some extent for biomethane production. Therefore, some of the factors that influence biomethane potential are the order of pretreatment and strain of fungi used. Since the aerobic fungi are seen to consume some carbohydrates for their growth during pretreatment, which leads to lower BMP, bioaugmentation with anaerobic fungi was studied in the next chapter. Anaerobic fungi have the advantage of shorter growth time compared to white-rot fungi and the loss of plant carbohydrates due to respiration can be avoided. This makes the usage of anaerobic fungi for biogas production very interesting.





## VII Perspectives of usage of anaerobic fungi for biogas production

### VII.1 Introduction

The digestive system of ruminant animals resembles the biogas fermenters with similar parameters like negative redox potential, a nearly neutral pH, and a temperature of  $37\pm 2^{\circ}\text{C}$ . In these natural biogas systems, anaerobic fungi play a major role in converting lignocellulosic biomass into energy by producing a plethora of plant carbohydrate hydrolyzing enzymes. These enzyme complexes are capable of separating the different fractions of lignocellulosic biomass. It is not only lignin that makes agricultural residues recalcitrant, but also other parameters such as cellulose crystallinity. The carbohydrate hydrolyzing enzyme mixture can degrade both amorphous and crystalline cellulose (Dollhofer et al., 2015). In the anaerobic digestion process, the maximum biodegradability of LCB is considered to be 60% and hydrolysis is considered as a rate-limiting step in this process (Proczka et al., 2012). The cellulolytic machinery of anaerobic fungi has received much attention in recent years, therefore making them promising candidates for improving the hydrolysis process (Dollhofer et al., 2015). Furthermore, the slurry obtained after anaerobic fungal mixed acid fermentation can directly be consumed by bacteria and methanogens involved in the AD process (Kazemi Shariat Panahi et al., 2022). However, their use for biogas production is now only gaining traction due to their high-maintenance and relatively unknown complex growth requirements (Vinzelj et al., 2020).

Anaerobic fungi were detected in seven out of ten agricultural biogas plants operated with a high share of cattle slurry or manure (Flad, 2020). *Feramyces austinii* is a strain isolated from wild Barbary sheep and fallow deer and characterized by Hanafy et al. (2018). To the author's knowledge, no study was carried out to investigate the potential of *Feramyces austinii* for bioaugmentation of anaerobic digestion even though *Feramyces* sp. have been identified in biogas plants. Therefore, the potential of *Feramyces austinii* to improve the biomethane yield of straw was estimated.

### VII.2 Materials and Methods

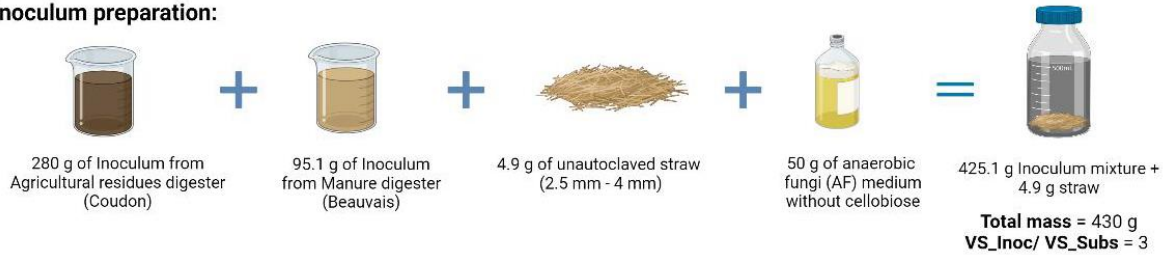
#### VII.2.1 Strain Maintenance

Anaerobic fungi *Feramyces austinii* was isolated by and obtained from the Institute of Microbiology, University of Innsbruck, Austria. It was subcultured weekly in the medium and protocol as given in Vinzelj et al. (2022) with cellobiose and incubated in stationary conditions at  $39^{\circ}\text{C}$  in serum bottles. The strain was viewed using an Olympus BX60 microscope attached to an Infinity 3 camera (Lumenera, Ottawa, ON, Canada).

## VII.2.2 Bioaugmentation Set-up

The BMP experiments were conducted in a setup as described in section IV.2.2.6.1. The inoculum for these experiments contained 75% of the digestate from a biogas plant (Coudon, France) treating agricultural waste and 25% of the digestate from a pilot reactor treating animal manure (UniLaSalle, Beauvais, France) for a total mass of 375 g. This was done as animal manure digestate could provide a similar natural rumen habitat for anaerobic fungi. But since the volatile solids (VS) content of animal manure digestate was low, digestate from agricultural biogas plants was added to increase the VS of inoculum. To remove large and undigested particles in the inoculum, they were filtered through a 1 mm porosity sieve and then left to stabilize for a week at room temperature. 50 mL of fresh media without cellobiose/straw as described in Vinzelj et al. (2022) was also added since we do not know if the inoculum satisfies the complex growth requirements for the anaerobic fungi to survive. Hereafter, 280 g of digestate from agricultural biogas plant added with 95.1 g of digestate from an animal manure biogas plant and 50 g of anaerobic fungal media would be referred to as an inoculum mixture. This inoculum mixture was used as negative control while cellulose with the inoculum mixture was used as positive control for BMP measurement. Meanwhile, multiple bottles of 50 mL subcultures of *Feramyces austinii* were prepared and let to grow for one week. They were then vacuum filtered under an anaerobic glove box and the total weight was measured. The anaerobic fungi were then divided into different quantities (0.89 g, 1.78 g, 4.9 g) based on their wet weight. The 4.9 g fresh weight was selected as the highest concentration to attempt since 4.9 g of straw (obtained from Hamiform in December 2021) was used for the biogas production study based on the ratio of VS substrate to inoculum. The different quantities of anaerobic fungi were added to each AMPTS bottle containing 4.9 g of straw (2.5 mm to 4 mm) with 425.1 g of inoculum mixture. An experimental control was run using 4.9 g of wheat straw, and 425.1 g of inoculum mixture. All the biogas experiments were carried out in duplicates in Automatic Methane Potential Test System (AMPTS) at 37°C for 51 days. The schematic representation of the methodology is given in Figure VII-1.

### Inoculum preparation:



### Controls in AMPTS:

#### Negative Control:

280 g of Coudon + 95.1 g of Beauvais + 50 g of AF media

#### Positive Control:

425.1 g of Inoc + 6.32 g of Cellulose

#### Experimental Control:

425.1 g of Inoc + 4.9 g of straw



### AMPTS



### Bioaugmentation protocol:

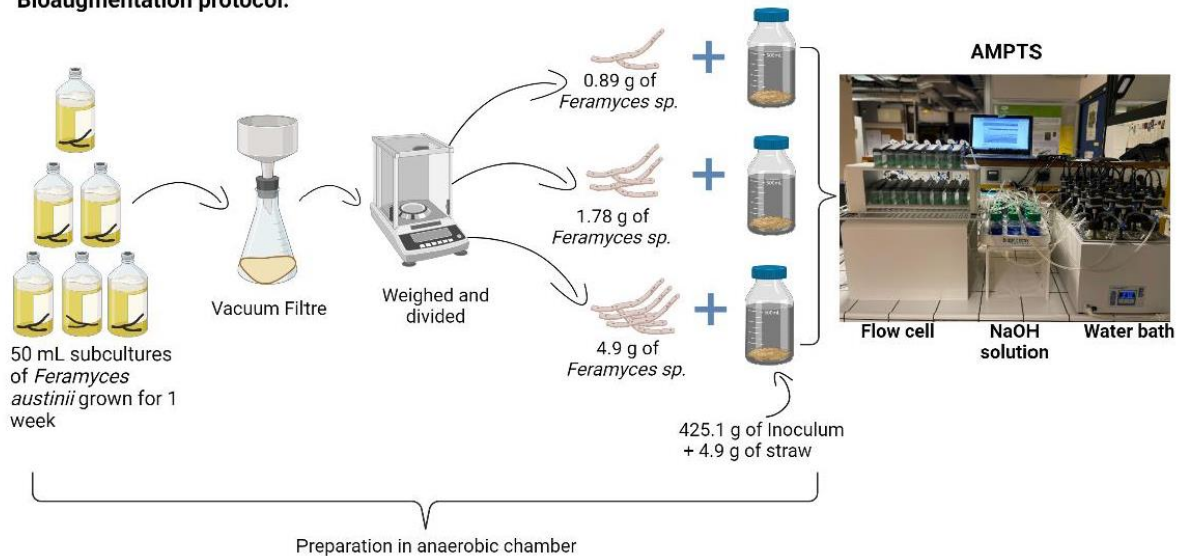


Figure VII-1: Schematic representation of bioaugmentation protocol

### VII.2.3 Lignin Composition

After the biomethane potential test was carried out for 51 days, the contents of the AMPTS bottles were sieved using a 300  $\mu\text{m}$  sieve. The obtained straw particles were thoroughly washed using demineralized water and dried at 40°C for 1-2 days. The lignin composition of raw straw before and after anaerobic digestion, and that of straw anaerobically digested in the presence of 4.9 g of anaerobic fungi were estimated according to the NREL protocol (Sluiter et al., 2012).

## VII.3 Results and Discussion

### VII.3.1 Strain Morphology

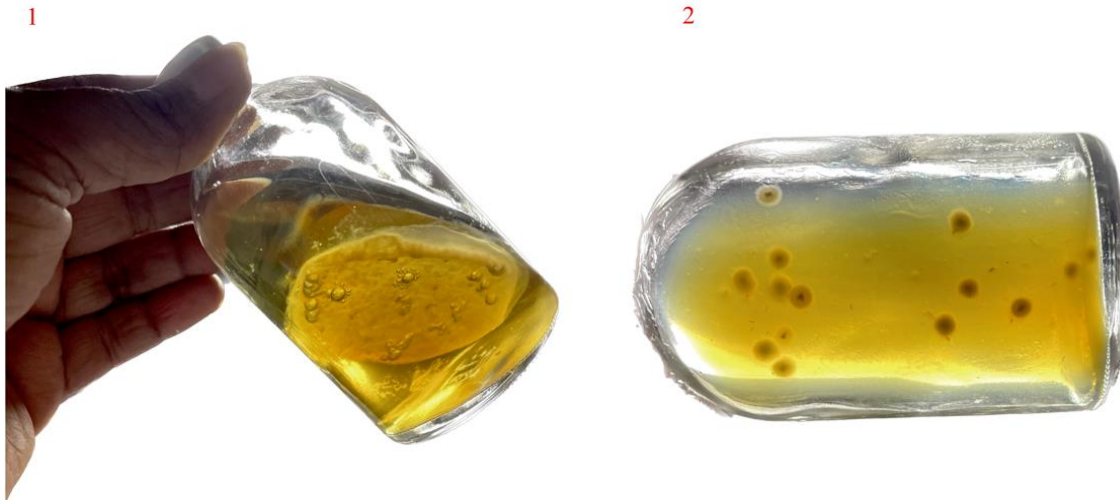


Figure VII-2: (1) Sub-culture of *Feramyces austinii* grown in liquid culture for a week.  
(2) Strain maintenance in roll agar tubes

As described in Hanafy et al. (2018), *Feramyces austinii* exhibited brown circular filamentous colonies of approximately 3 mm with a darker brown central core when cultured in roll agar tubes (refer to Figure VII-2 (2)). In liquid cultures, it formed a large, thick growth on the surface of the tubes which loosened and floated to the surface of the liquid culture when shaken (refer to Figure VII-2 (1)). In Figure VII-3 (B), a filamentous rhizoidal system and a monocentric thallus with zoospores are visible.

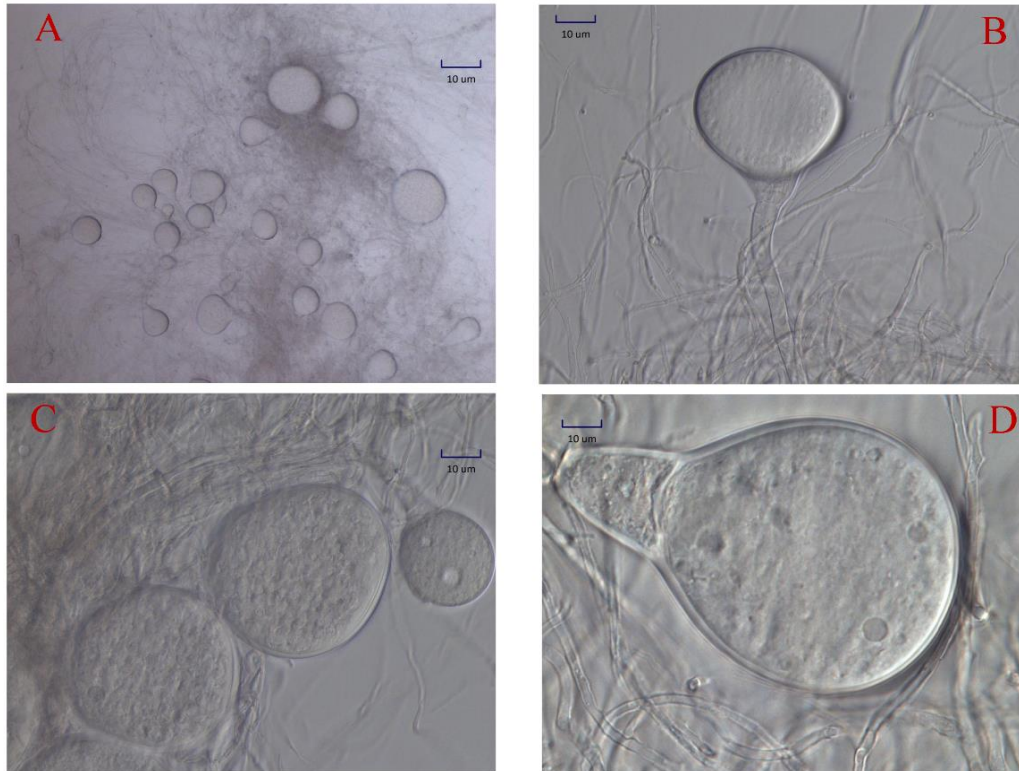


Figure VII-3: Microscopy images of *Feramyces austinii* taken at (A) 10x magnification, (B) 40x magnification, (C) 100x magnification, and (D) phase

### VII.3.2 Biomethane Potential Test

As can be observed from Figure VII-4, no improvement in biomethane potential is observed for straw samples bioaugmented with low quantities (0.89 g and 1.78 g) of anaerobic fungi as compared to the experimental control. This is probably because low quantities of anaerobic fungi could not establish themselves within the competitive environment of the AD reactors and therefore do not have higher biomethane yield than experimental control. Bioaugmentation with 4.9 g of anaerobic fungi showed the highest biomethane yield of  $351.82 \pm 12.35 \text{ NL}_{\text{CH}_4}/\text{kg}_{\text{VS}}$  which is 7.2% higher than the experimental control (Figure VII-4). Since 4.9 g is the fresh weight of anaerobic fungal biomass used, the actual dry weight of the biomass is quite less compared to the billion microorganisms found in the AD system (375 g of inoculum). Therefore, even a 7.2% increase in BMP is quite substantial. In a study by Nkemka et al. (2015), bioaugmentation carried out with 10% (v/v) of *Piromyces rhizinflata* did not improve the biomethane output of corn silage or cattail. However, it improved the initial production rates which helps to reduce the digestion time (Nkemka et al., 2015). In another study where bioaugmentation was carried out with 10% (v/v) *Orpinomyces* sp. in a co-digestion system (different types of straw with cow manure) enhancement of the methane yield between 15 and 23% was observed (Akyol et al., 2019). Procházka et al. (2012) found that depending

on the substrate and anaerobic fungi species used, the biogas production varied from 4 – 22% when using approximately 8 mg of dry mycelium.

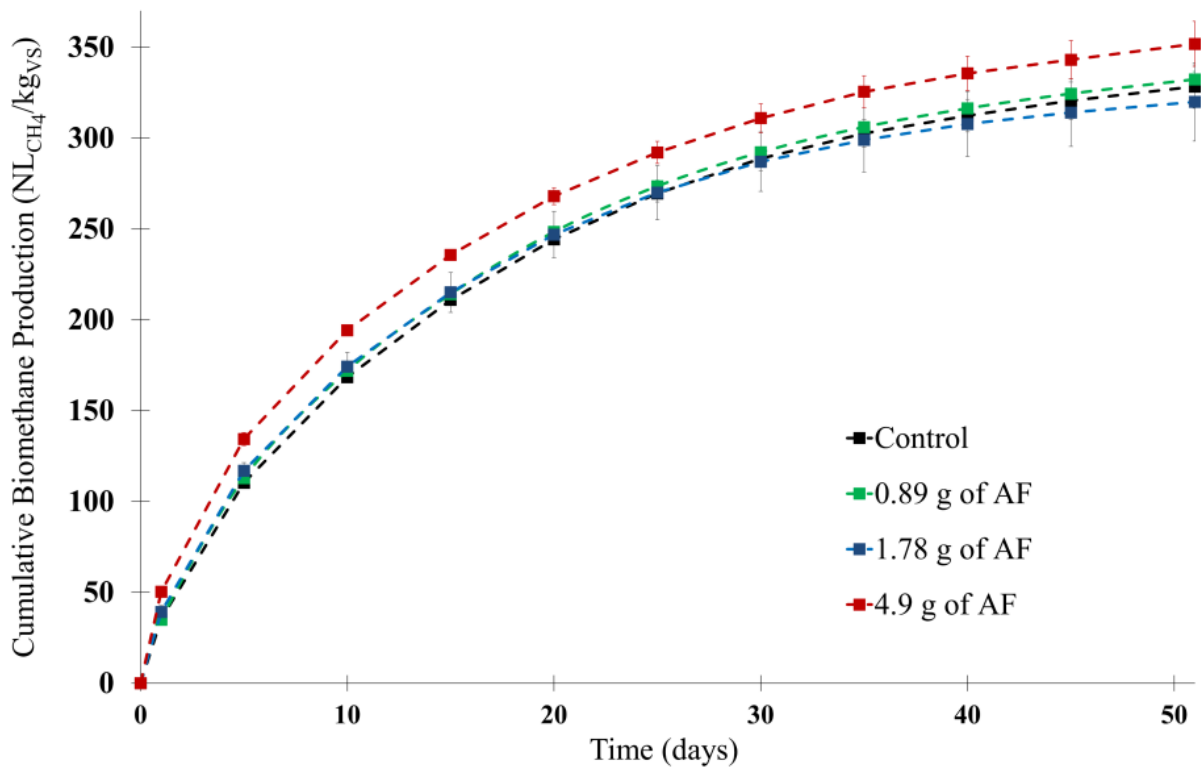


Figure VII-4: Cumulative biomethane potential (NL<sub>CH<sub>4</sub></sub>/kg<sub>vs</sub>) over time for bioaugmented straw with *Feramyces austinii*

Non-linear regression was carried out using Scilab (Dassault Systèmes, Vélizy-Villacoublay, France) in order to extract the kinetic parameters ( $k$  and  $V_{max}$ ) of the first-order model. The values of the first-order kinetic constants obtained from the best fit of the data of Figure VII-4 along with the correlation coefficient ( $R^2$ ) values are reported in Table VII-1. It can be observed that the kinetics ( $k$  ( $d^{-1}$ )) improved with increasing quantity of anaerobic fungi. Bioaugmentation with 0.89 g of anaerobic fungi was able to improve the methanization kinetics ( $0.086 d^{-1}$ ) at the beginning but not the global production yield. This is probably because of the inability of the anaerobic fungi to survive in the anaerobic digester for a long time when supplemented in small quantities. Whereas 4.9 g of anaerobic fungi exhibited a better hydrolysis rate ( $0.094 d^{-1}$ ) which helps to reduce the digestion time and improve the overall yield by maintaining a stimulating action for a longer methanization period.

**Table VII-1:** Parameters of first-order kinetics

Sample	Vmax (NL <sub>CH<sub>4</sub></sub> /kg <sub>vs</sub> )	k ( $d^{-1}$ )	R <sup>2</sup> (-)
Experimental Control	316.3	0.077	0.995

Bioaugmentation_0.89 g	318.7	0.078	0.996
Bioaugmentation_1.78 g	305.7	0.086	0.994
Bioaugmentation_4.9 g	323.3	0.094	0.989

### VII.3.3 Lignin Composition

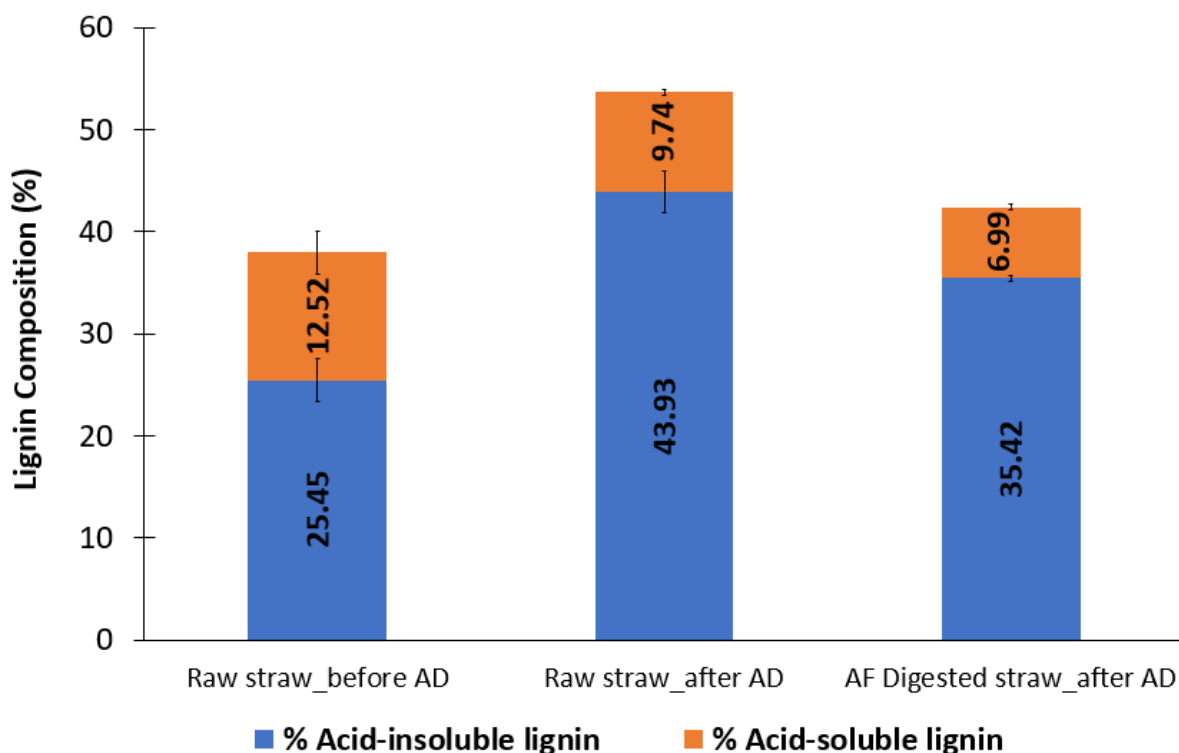


Figure VII-5: Lignin composition of raw straw before and after anaerobic digestion with and without bioaugmentation with anaerobic fungi

It can be observed from Figure VII-5 that the lignin content of raw straw increased by 58.66% after anaerobic digestion. This is because the carbohydrates are used up in the bioconversion process to CH<sub>4</sub> and CO<sub>2</sub>. Thereby, the relative proportion of undegraded lignin increases. Whereas, the lignin content of the straw anaerobically digested in the presence of 4.9 g of *Feramyces austinii* has only increased by 25.41%. This is probably because of the synergy of anaerobic fungi with methanogens, which helped to mineralize lignin. From Figure VII-4 and Figure VII-5, it could be assumed that the lignin mineralization in anaerobic conditions could have led to better methane yield when bioaugmented with 4.9 g of anaerobic fungi.

Anaerobic fungi cannot catabolize lignin like white-rot fungi which have an aerobic enzymatic machinery to cleave aromatic rings. Anaerobic fungi, specifically, break the plant fibers by growing and expanding their rhizoids. This process helps to separate lignin and makes hemicellulose and lignin vulnerable to further enzymatic digestion (Flad, 2020). They possess



a symbiotic relationship with archaea based on the inter-species hydrogen transfer effect. This relationship helps in the formation of acetate and formate which are preferred growth substrates for methanogens. This process helps to reduce the accumulation of VFAs which cause digester failure. Fungi- archaea symbiotic relationship is so vital that some rumen fungi can never be isolated in pure culture. Previous studies have shown the close relationship between anaerobic fungi – methanogens, such as co-cultures of *N. frontalis* + *M. gottschalkii* YakQH5 which can decompose lignin efficiently (Wei et al., 2022). Methanogenesis is an important microbial metabolism in various natural anaerobic environments such as soil and freshwater/ marine sediments. Some studies have shown the potential of anaerobic bacteria to degrade lignin under anaerobic conditions. But the knowledge of the microorganisms and their mechanism of degradation of lignin-derived aromatics is very limited (Y. Li et al., 2021). Further analysis of the distribution of methanogens in the anaerobic digesters are required to confirm the synergy of *Feramyces austinii* with methanogens.

#### **VII.4 Conclusion and a perspective summary of Chapter VII**

It was observed that bioaugmentation with 1.78 g of *Feramyces austinii* improves the methanization kinetics at the beginning but not the global production yield whereas 4.9 g of inoculated anaerobic fungi seems to both improve the production rate and the global yield by maintaining a stimulating action for a longer methanization period. The synergy of anaerobic fungi and methanogens to degrade lignin in anaerobic conditions is an interesting field of study that would help to strengthen research for the use of LCBs in large-scale industrial production of bioenergy. Even though anaerobic fungi application in AD is seen to be clearly beneficial for improving the methane yield, their viability must be improved. This could be addressed by genetic engineering techniques to prevent anaerobic fungi to be suppressed within the competitive environment of biogas reactors. Bioaugmentation of anaerobic digesters is restricted to the lab scale because of their unknown complex growth requirements for their long-term survival in reactors. Genetically engineered strains, investigation of syntrophic co-cultures with methanogens, and new reactor configurations would help to successfully scale up the bioaugmentation processes (Kazemi Shariat Panahi et al., 2022). The results obtained in this study are a step in the direction to investigate the feasibility and indicate the promising approach of bioaugmentation of biogas reactors with *Feramyces austinii*.

## VIII General Conclusions and Future Scope

The work was conducted aiming to better understand the efficiency of combined pretreatment of straw for biogas production. In this context, both physical-chemical and chemical-biological pretreatment were studied for their impact on biomass characteristics, and biomethane potential. Physical pretreatment was conducted by grinding and autoclaving because they are commonly used techniques in combination with other pretreatment methods. For the chemical pretreatment, Fenton pretreatment which is a process of non-selective degradation of organic compounds was chosen as it mimics the wood decay by fungi. Aerobic fungal pretreatment with *Pleurotus ostreatus* is a well-studied process and is known to efficiently degrade various substrates. It uses an oxidative enzymatic mechanism to metabolize the main constituents of the biomass. Since it is a widely produced variety of mushrooms, called oyster mushrooms, there is an interest to use the residues of mushroom cultivation along with the mycelium for biogas production. On the other hand, anaerobic fungi which in the last decade is gaining traction for their cellulolytic machinery were also studied for their effect on enhancing biogas production.

### Main results

Fenton pretreatment was studied with three different concentrations of  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  for its ability to reduce the recalcitrant nature of biomass. It was observed that low concentrations of Fenton reagents ( $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$ ) were effective enough to cause lignin degradation and reduce the crystallinity of the biomass. However, it is not effective enough to cause a significant increase in the biomethane potential. Fenton pretreatment was studied in combination with physical pretreatments such as grinding and autoclaving. Size reduction was observed to be a strong pretreatment method that masked the effect of Fenton pretreatment. On the other hand, autoclaving helped to slightly improve the kinetics but not the overall biomethane yield. When their synergies were studied together, it caused a significant increase in the biomethane yield. However, physical pretreatment is an energy-intensive process and therefore, studying low-cost alternatives like biological-chemical combined pretreatment is important.

Spent mushroom substrate which poses a challenge of disposal for the mushroom industry, was studied as a source for biogas production. Wheat straw substrates were obtained at different stages of harvest from two hybrid strains (HK35 and Spoppo) of oyster mushrooms. It was observed that the strain of fungi influenced the biogas yield. The spent mushroom wheat straw obtained after 2 flushes of *Pleurotus ostreatus* HK35 was the best substrate for biogas production as compared to the Spoppo strain. For the mushroom industry, it is more resourceful

to harvest 2 rounds of mushrooms using the same substrate and then to use the well-degraded substrate for integrated energy production. Therefore, to compensate for the high production cost of mushrooms, bioenergy production from spent substrate could help to counteract some energy costs.

Solid-state cultivation of fungi, such as in the case of mushroom production, has the disadvantage of a slow growth rate of fungi because the environmental conditions of microbes cannot be regulated. Submerged cultivation, on the other hand, provides better homogeneity of nutrients and better oxygen transfer. Three pure strains of *Pleurotus ostreatus*, namely, MUCL 20510, MUCL 28511, and MUCL 29420 from the mycology bank were studied for their biomass degrading efficiency in submerged conditions. Again, variation among the strains for the bioprocessing capabilities was observed. The variations among the strains of the same fungal species can be attributed to their growth characteristics, genetic sequences, and enzyme profiles. Moreover, the same strain varied in the degradation ability based on the cultivation conditions, such as solid-state or submerged cultivation which influence several parameters such as incubation time, pH, aeration, etc. Therefore, there is a need to screen a large number of fungal isolates to choose the right strain for the particular pretreatment process.

In general, sole fungal treatment with *Pleurotus ostreatus* MUCL 29420 strain was seen to improve the BMP whereas *Pleurotus ostreatus* HK35 strain did not show any improvement in submerged conditions. Combining biological-chemical pretreatment was also beneficial only based on the strain used. Altering the order of sequence of the combined pretreatment is seen to affect the solubilization of the biomass. The difference is due to the mechanism of action based on the order of application which differed in the type of released substances after each step. In general, chemical pretreatment followed by fungal pretreatment (CP+FP) was observed not to be beneficial for anaerobic digestion. This was because chemical pretreatment made the inaccessible polysaccharides more accessible for subsequent fungal pretreatment. This led to the utilization of the released substances for their growth and thereby, lower biomethane yield. The combined pretreatment of fungal followed by the chemical method (FP+CP) was seen to be beneficial for the HK35 strain whereas, it did not improve the biogas production for the PO3 strain as compared to fungal pretreatment alone. Therefore, the efficiency of combined biological-chemical pretreatment is also strain specific. The order of pretreatment is also very important as it affects the mechanism of action on the biomass which affects the biogas production.

Bioaugmentation with anaerobic fungi is a relatively new and exciting field of research to improve biogas production. The production of anaerobic fungi was quite challenging due to

its high maintenance. *Feramyces austinii* was seen to improve the hydrolysis rate and the global methane production of straw. Delignification of lignocellulose by anaerobic fungi was observed which provides holocellulose more susceptible to bacterial degradation in anaerobic conditions. Further genotyping studies and enzymatic studies could help to explain the symbiotic relationships of *Feramyces austinii* and its ability to degrade lignin in anaerobic conditions.

### **Limitations and prospects**

Fungal pretreatment is an environmental-friendly method and is seen to be efficient for biogas production from certain strains and cultivation conditions. The differences in the degradation ability between the strains of the same fungal species make it challenging for technology transfer to a large scale. Therefore, genomic and enzymatic analysis of different isolates needs to be carried out to be better able to choose the right strain for study. Nevertheless, apart from the strain of fungi, pretreatment conditions, and species of biomass also affect biogas production. Therefore, a multivariate analysis of combined pretreatment can help to further optimize the pretreatment conditions. Anaerobic fungi open a vast area of research for their synergistic relationship with methanogens and their enzymatic action. Though this research emphasizes the need to exploit the unexplored anaerobic fungi for biogas production, cultivation and maintenance of these of it is still a huge challenge. There is a need to fully develop cultivation techniques for large-scale production of these fungi before they can be widely used to improve biogas production. The study of the synergy between anaerobic fungi and methanogens can also provide knowledge on the type of inoculum to be used for bio-augmentation protocols. The intricacy of the lignocellulosic matrix and heterogeneity in the physicochemical composition of the biomass makes it complex to study. The outcome of various pretreatment methods applied to the biomass is not sufficiently described by the currently available state-of-the-art characterization techniques. Consequently, current pretreatment studies can only be considered in case-by-case studies. Therefore, further improvement and development of analytical techniques are needed to be able to understand the structural and chemical changes that biomass undergoes during pretreatment. This will help to develop models to predict the biomethane potential of a feedstock. Further, energy and cost analysis of the different pretreatment needs to be carried out to better able to estimate the technology transfer readiness level.



## **IX Summary in French**

### **IX.1 Introduction Générale**

La crise énergétique mondiale de ces derniers temps a eu un impact sans précédent qui se fera sentir dans les années à venir. Elle a mis en évidence la nécessité d'accélérer la transition vers un système énergétique plus durable et plus sûr. Le mouvement en faveur de la résilience énergétique s'accompagne d'un changement de paradigme en faveur des énergies renouvelables (Agence internationale de l'énergie, 2022). Le nombre de gouvernements ayant pris des engagements ambitieux en matière d'émissions nettes nulles d'ici à 2050 a rapidement augmenté. Cependant, malgré la croissance de l'électricité à faible teneur en carbone, la plupart des secteurs du transport maritime, de l'aviation et de certains secteurs industriels ne sont pas encore "prêts pour l'électricité" et ont besoin d'autres sources d'énergie. C'est pourquoi le biogaz et le biométhane sont de plus en plus demandés pour s'imposer dans la consommation mondiale d'énergie. En 2018, la consommation directe mondiale de biogaz était d'environ 35 Mtep (millions de tonnes d'équivalent pétrole), l'Europe étant la première région productrice de biogaz. Actuellement, 70 % du biométhane utilisé en Europe provient de cultures énergétiques. Par conséquent, les politiques qui encouragent l'utilisation des déchets de culture gagnent du terrain afin de décourager le débat nourriture/carburant présent lors de l'utilisation des cultures énergétiques. On prévoit que la consommation directe de biogaz atteindra environ 75 Mtep en 2040. La majeure partie de ce biogaz sera produite par des usines centralisées alimentées par des déchets solides agricoles et municipaux afin de répondre à la demande locale d'électricité et de chauffage. Il existe également une motivation considérable pour développer le biogaz pour la cuisson propre en Afrique et en Inde. La production de biogaz permet non seulement de répondre aux besoins énergétiques, mais aussi d'améliorer les pratiques de gestion des déchets (déchets solides municipaux, boues d'épuration, résidus de culture, fumier animal) et de réduire la pollution atmosphérique (due au brûlage de chaume et à l'utilisation du charbon), en particulier dans les pays en développement. En outre, la valorisation du biogaz en biométhane pour l'utiliser comme carburant augmente en Europe et en Amérique du Nord. L'avantage distinctif du biométhane par rapport au bioéthanol et au biodiesel repose sur les limitations de la part de mélange, car le bioéthanol et le biodiesel ne sont pas identiques à l'essence et au diesel. Cependant, le biométhane peut remplacer totalement le gaz naturel comme source de carburant sans aucune modification du moteur. Par conséquent, la transformation de déchets organiques tels que les résidus agricoles en produits de plus grande valeur tels que le biogaz et le biométhane s'inscrit parfaitement dans le concept d'économie circulaire (Agence internationale de l'énergie, 2020).

Bien que la bioénergie soit une énergie renouvelable prometteuse, son déploiement se heurte à certains obstacles. Les incertitudes politiques et le manque de sensibilisation aux produits bioénergétiques sont quelques-uns des facteurs qui réduisent la demande du marché. Les obstacles financiers et économiques qui accompagnent les structures institutionnelles complexes sont les subventions accordées aux combustibles fossiles, qui rendent la bioénergie moins compétitive sur le marché. Par conséquent, les politiques de tarification du carbone et les subventions à la bioénergie peuvent rendre cette dernière plus abordable. Les principaux facteurs qui entravent la transition sont la faiblesse des chaînes d'approvisionnement et le faible niveau de préparation technologique. L'instabilité de l'approvisionnement en matières premières due au manque d'informations sur la disponibilité et la localisation des ressources bioénergétiques est à l'origine de la faiblesse des chaînes d'approvisionnement. La faiblesse des réglementations en matière de contrôle de la qualité et de normalisation, le manque d'infrastructures et l'absence de recherche sur la production de bioénergie à grande échelle sont les causes d'un faible niveau de préparation technologique. Par conséquent, une bonne combinaison de politiques, de recherche, de développement et de démonstration peut contribuer à accélérer la commercialisation de la bioénergie (Feng, 2022).

La digestion anaérobie (DA) est un processus biologique qui transforme les matières organiques, telles que les cultures énergétiques, les résidus agricoles et forestiers, les boues d'épuration et les déchets animaux et alimentaires, en biogaz (principalement du méthane et du dioxyde de carbone). La digestion assistée se produit en l'absence d'oxygène avec l'aide de micro-organismes et se compose généralement de plusieurs étapes. La première étape est l'hydrolyse, au cours de laquelle les molécules organiques complexes sont décomposées en sucres plus simples par les enzymes sécrétées par les micro-organismes. Au cours de la deuxième étape, l'acidogénèse, les sucres sont convertis en acides organiques tels que l'acide acétique et l'acide propionique par des bactéries acidifiantes. Vient ensuite l'acétogénèse, au cours de laquelle les acides organiques sont convertis en acétate, en hydrogène et en dioxyde de carbone par des bactéries acétogènes. Enfin, la quatrième étape est la méthanogénèse où l'acétate, l'hydrogène et le dioxyde de carbone sont convertis en méthane et en dioxyde de carbone par des bactéries méthanogènes (Uçkun Kiran et al., 2016). Parmi les matières organiques disponibles, la biomasse lignocellulosique (LCB) est la ressource renouvelable la plus abondante et la moins chère sur Terre. Environ  $120 \times 10^9$  tonnes de LCB sont produites chaque année dans le monde, ce qui permettrait de produire  $2,2 \times 10^{21}$  Joule d'énergie, soit 300 fois plus que les besoins énergétiques mondiaux actuels (Guo et al., 2015). La production de biométhane à partir de LCB est considérée comme un moyen efficace de production d'énergie en raison d'un rapport énergie produite/entrée plus élevé (Abraham et al., 2020).

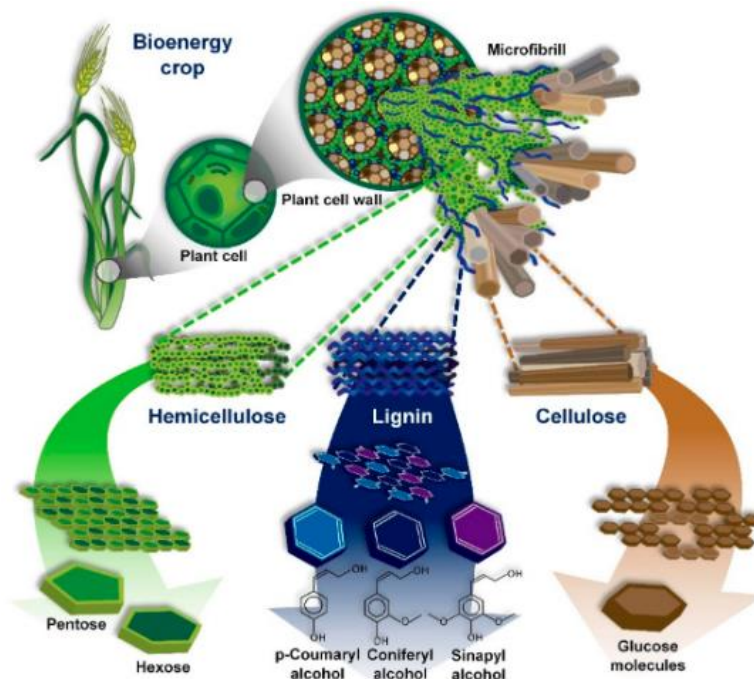


Figure IX-1: Structure de la biomasse lignocellulosique et de ses biopolymères (Réutilisation (impression) avec la permission de Hernández-Beltrán et al. (2019) (licence Creative Commons Attribution (CC BY))).

La lignocellulose est la principale composante des parois cellulaires des plantes et se compose de trois macromolécules principales, à savoir la lignine, l'hémicellulose et la cellulose (comme le montre la Figure IX-1). Ces trois composants forment une structure tridimensionnelle complexe et résistante grâce à des liaisons covalentes et hydrogène. La proportion de chaque composant varie pour chaque biomasse (plantes herbacées, tiges et feuilles de céréales, etc.) et dépend également de l'origine (source, espèce, conditions climatiques, etc.) (Alfenore et Molina-Jouve, 2016 ; Anukam et Berghel, 2021). L'un des principaux résidus agricoles est la paille, puisqu'environ 35 millions de tonnes de blé sont produites par an en France et qu'environ 0,85 kg de paille est généré par kg de blé récolté (Zhang et al., 2022). Les caractéristiques inhérentes à la biomasse, telles qu'un taux d'humidité élevé, une faible densité énergétique, une faible densité apparente, une forme et une taille irrégulières, la rendent difficile à utiliser sous sa forme naturelle pour la production d'énergie. En outre, la complexité structurelle et l'hétérogénéité de la biomasse, la nature cristalline de sa teneur en cellulose et l'étendue de la lignification sont responsables de la nature récalcitrante de la biomasse. Pour une meilleure exploitation de la biomasse, une étape de prétraitement est essentielle pour surmonter sa récalcitrance, qui est principalement causée par la lignine. L'étape de prétraitement permet de désagréger la lignine des composants de la cellulose et de l'hémicellulose (comme le montre la Figure IX-2). Cela rend la cellulose accessible et augmente la surface d'attaque microbienne et enzymatique, ce qui prépare la biomasse à une récupération



maximale du produit. Il existe plusieurs méthodes de prétraitement, notamment des méthodes physiques, chimiques, physico-chimiques et biologiques. Le choix du prétraitement dépend du type de LCB utilisé et du processus en aval (Alfenore et Molina-Jouve, 2016 ; Anukam et Berghel, 2021).

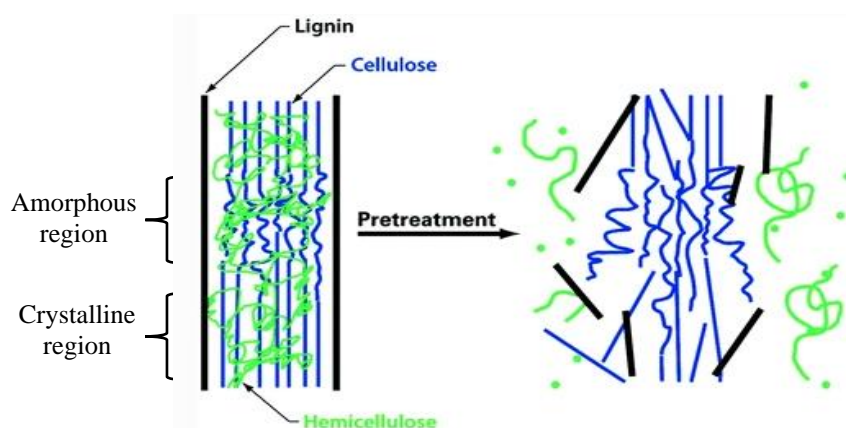


Figure IX-2: Représentation schématique du prétraitement de la biomasse lignocellulosique (Image adaptée pour la réutilisation (impression) avec la permission de Ji et al. (2011) (Copyright Springer Nature)).

Le prétraitement physique est inévitable avant la conversion biochimique et/ou thermochimique de la biomasse afin d'éliminer les limitations de transfert de masse et de chaleur. Il améliore l'accessibilité aux polysaccharides de la biomasse et augmente la surface d'attaque microbienne. Cependant, il existe peu d'informations sur la manière dont le prétraitement physique modifie la structure ou la composition chimique de la biomasse. Certains des inconvénients du prétraitement physique sont son manque de capacité à se débarrasser de la lignine et sa forte consommation d'énergie. Les méthodes physiques courantes comprennent le broyage et la mouture, tandis que les méthodes chimiques courantes comprennent les traitements à l'acide, à l'alcali et à l'oxydant. Le prétraitement chimique implique l'utilisation de composés organiques ou inorganiques pour perturber la structure de la biomasse en interagissant avec les liaisons intra- et inter-polymères des composés organiques primaires. Le prétraitement chimique est une méthode couramment utilisée en raison de sa grande efficacité. Néanmoins, il peut avoir un impact significatif sur le processus en aval en raison de la forte concentration des réactifs chimiques utilisés. En outre, des processus supplémentaires sont nécessaires pour recycler les réactifs utilisés, faute de quoi les résidus pourraient entraîner une pollution de l'environnement. Le prétraitement physico-chimique est une approche combinée, réalisée sur une large gamme de températures (de 50 à 250 °C) pour rompre les liaisons hydrogène entre les polymères complexes à l'aide de la chaleur. L'utilisation des flux de chaleur résiduelle est importante pour une gestion efficace de l'énergie. Le temps de prétraitement dans le processus physico-chimique doit être optimisé car une exposition

prolongée à la chaleur peut entraîner des réactions indésirables et la formation d'inhibiteurs du processus de DA. D'autre part, le prétraitement biologique est une approche peu coûteuse et respectueuse de l'environnement, car il implique l'utilisation d'enzymes ou de micro-organismes pour décomposer la biomasse. Il nécessite moins d'énergie et des conditions de traitement douces. Il contribue à la délignification, à la décomposition de l'hémicellulose et à la réduction de la cristallinité de la cellulose. L'inconvénient du prétraitement biologique est le temps de séjour plus long et l'efficacité plus faible (Abraham et al., 2020 ; Anukam et Berghel, 2021 ; Mankar et al., 2021).

Bien que la lignine soit un énorme réservoir naturel de carbone et d'énergie, seul un petit groupe de champignons basidiomycètes filamenteux appelés champignons de la pourriture blanche (WRF) a développé la capacité de dépolymériser et de minéraliser efficacement la lignine en CO<sub>2</sub> et en H<sub>2</sub>O. Les WRF utilisent des systèmes d'oxydoréductase ligninolytique extracellulaires, tels que les laccases et les peroxydases, ainsi que d'autres métabolites sécrétés pour la dépolymérisation de la lignine (del Cerro et al., 2021). Cependant, la porosité des LCB est faible par rapport à la taille des enzymes ligninolytiques, qui ne peuvent donc pas pénétrer dans la paroi cellulaire. Par conséquent, les champignons de délignification utilisent un système non enzymatique appelé réaction de Fenton médiée par un chélateur pour effectuer la dégradation initiale, car les espèces réactives de l'oxygène ont un faible poids moléculaire et peuvent pénétrer la paroi cellulaire (Hashemi et al., 2022). *Pleurotus ostreatus*, communément appelé pleurote, est un WRF largement cultivé pour sa grande valeur nutritionnelle et médicinale. Il peut être cultivé sur une large gamme de substrats et les résidus de mycélium obtenus après la récolte du champignon comestible sont appelés substrat de champignon usagé. Cependant, le prétraitement fongique a un temps de réaction long et une faible efficacité. Par conséquent, une combinaison avec un prétraitement chimique qui imite la dégradation des parois cellulaires des plantes par l'action des champignons pourrait améliorer l'efficacité du prétraitement fongique. Le prétraitement Fenton est un processus d'oxydation-réduction du fer par H<sub>2</sub>O<sub>2</sub>. Il s'agit d'une réaction cyclique et les radicaux hydroxyles réactifs générés peuvent contribuer à la dégradation de la paroi cellulaire végétale. Le prétraitement fongique nécessite très peu d'énergie, tandis que le prétraitement chimique utilisé en combinaison ne nécessiterait que des conditions d'exploitation modérées, la combinaison améliorant la productivité globale du processus (Hashemi et al., 2022 ; Shirkavand et al., 2016). Les champignons aérobies utilisent des hydrates de carbone pour leur respiration et leur processus de prétraitement est assez long. En revanche, les champignons anaérobies peuvent empêcher la perte d'hydrates de carbone due à la respiration (Vinzelj et al., 2020).

Les champignons anaérobies produisent des enzymes cellulolytiques capables de couper les liaisons ester qui relie l'hémicellulose et la lignine. Ils ont des relations synergiques et antagonistes avec d'autres organismes tels que les méthanogènes qui peuvent consommer les produits intermédiaires. Par conséquent, la bioaugmentation du système de DA avec des champignons anaérobies peut augmenter la production de méthane (Vinzelj et al., 2020). Plusieurs genres tels que *Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Cyllamyces*, *Piromyces*, *Anaeromyces* et *Feramyces* ont été détectés dans des usines de biomasse agricole contenant une part importante de lisier de bovins. Cela montre que les champignons anaérobies peuvent être présents et transcriptionnellement actifs dans les usines de biogaz, mais avec des restrictions substantielles. Étant donné que leur activité métabolique dans les usines de biogaz est peu intense, on ne sait pas exactement quels paramètres entravent leur activité dans les DA (Flad, 2020). Certaines études de bioaugmentation ont déjà été réalisées en utilisant *Neocallimastix*, *Orpinomyces*, *Piromyces* et *Anaeromyces* pour la production de biogaz. *Feramyces austinii* est une souche récemment isolée à partir de moutons sauvages de Barbarie et de daims. Il a été démontré qu'elle avait une croissance rapide et robuste sur la biomasse végétale et la capacité de métaboliser une large gamme de mono-, oligo- et polysaccharides (Hanafy et al., 2018). Par conséquent, ces organismes relativement peu explorés sont intéressants pour étudier leur potentiel de production de biogaz. Cependant, ils représentent un défi considérable pour la mise à l'échelle du processus de production en raison de leurs exigences de croissance complexes encore inconnues (Kazemi Shariat Panahi et al., 2022 ; Vinzelj et al., 2020).

Il est également important de déterminer la quintessence des propriétés physicochimiques de la biomasse afin de mieux comprendre dans quelle mesure le matériau se prête à la conversion et l'effet du prétraitement sur la biomasse. En comprenant les changements structurels de la biomasse, il est possible de déterminer le mode d'action du prétraitement pour augmenter le rendement en biogaz. Certains des facteurs importants d'un prétraitement réussi sont la dégradation de la lignine, la modification du degré de polymérisation de la cellulose et l'accessibilité des enzymes. Il est donc impossible d'étudier l'effet du prétraitement avec un seul facteur. Néanmoins, l'hétérogénéité est une caractéristique inhérente à la biomasse. Par conséquent, une grande variété de techniques analytiques de pointe est nécessaire pour interpréter les caractéristiques de la biomasse avant et après le prétraitement. Chaque technique analytique présente des inconvénients spécifiques qui rendent difficile la prédiction exacte de l'efficacité d'un prétraitement (Anukam et Berghel, 2021 ; Karimi et Taherzadeh, 2016). Par conséquent, il est important d'effectuer une digestion anaérobie ou une saccharification enzymatique pour comprendre l'effet du prétraitement sur le rendement du biocarburant souhaité.

## IX.2 Les objectifs de recherche et approche scientifique

Dans cette thèse, l'effet du prétraitement individuel et combiné de la paille a été étudié pour sa capacité à améliorer la production de biogaz. La paille a été utilisée pour cette étude car il s'agit d'une biomasse abondante et bon marché. Une bibliographie détaillée des différents prétraitements fongiques et chimiques a été réalisée sur la base de leur efficacité pour la dégradation des fibres, la production d'hydrates de carbone, les rendements en biogaz et en bioéthanol. Cela a permis de choisir les conditions et les stratégies de prétraitement à utiliser. L'approche scientifique pour atteindre cet objectif a consisté à étudier d'abord les étapes de prétraitement fongique et chimique individuellement et à choisir les conditions optimales pour le prétraitement combinatoire. Pour le prétraitement biologique, *Pleurotus ostreatus* a été choisi car 2500 tonnes de ce champignon sont produites chaque année en France pour la consommation. Par conséquent, les substrats de champignons usagés constituent une ressource précieuse pour la production d'énergie. Le processus de production des champignons s'effectue par culture en milieu solide. Même si la culture en milieu solide fournit un environnement auquel les champignons filamenteux sont naturellement adaptés, la culture en milieu immergé fournit un environnement plus homogène. L'un des objectifs de ce travail était donc d'évaluer la production de biogaz après un prétraitement fongique en culture en milieu solide et en culture submergée. Différentes souches de *Pleurotus ostreatus* ont été utilisées pour cette étude, à savoir des souches hybrides provenant de l'industrie du champignon et des souches pures provenant de la banque de mycologie. Pour le prétraitement chimique, le prétraitement oxydant utilisant  $\text{Fe}^{2+}$  et  $\text{H}_2\text{O}_2$  a été étudié car il imite la réaction de type Fenton naturellement utilisée par les champignons de la pourriture blanche. En général, les prétraitements physiques tels que le broyage et l'autoclavage sont effectués avant tout autre prétraitement. Par conséquent, leur effet sur la structure de la paille et la production de biogaz a également été estimé. Un large éventail de techniques analytiques a été utilisé pour caractériser la biomasse avant et après le prétraitement afin d'établir la corrélation entre les caractéristiques de la biomasse et sa production de biométhane. Des recherches préliminaires ont également été menées sur la bioaugmentation avec des champignons anaérobies. La souche choisie, *Feromyces austinii*, n'a été isolée et caractérisée que récemment et il s'agit donc de l'une des premières études sur l'utilisation de cette souche pour l'amélioration de l'AD. Le schéma général du projet est présenté à la Figure IX-3.

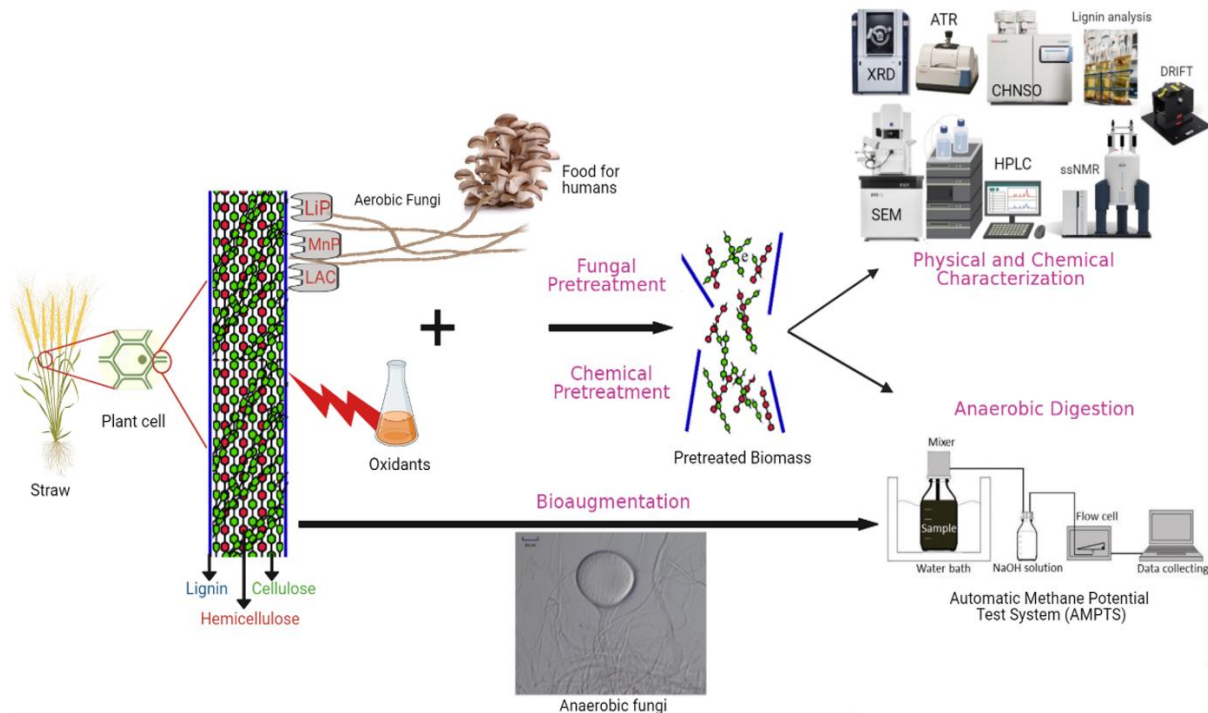


Figure IX-3: Représentation schématique de l'ensemble du projet. Créée avec BioRender.com

L'approche scientifique de cette thèse a consisté en plusieurs objectifs plus modestes, comme indiqué ci-dessous :

1. Un examen approfondi du prétraitement biologique-chimique combiné du LCB basé sur sa capacité à dégrader les fibres, la production de sucre, le rendement en biogaz et le rendement en bioéthanol. Une revue des différentes techniques analytiques utilisées pour la caractérisation de la biomasse.
2. Optimisation de la concentration des oxydants pour le prétraitement de la paille sur la base de la littérature.
3. L'effet de la taille des particules, du processus d'autoclavage et de la réaction de Fenton sur la production de biométhane à partir de la paille.
4. Comparaison de deux substrats de champignons épuisés avant et après différentes étapes de récolte pour la production de biogaz afin de permettre une approche "déchets-énergie" pour les industries du champignon.
5. Comparaison de différentes souches de *Pleurotus ostreatus* obtenues à partir de la banque de mycologie sur la base de la dégradabilité de la biomasse dans des conditions d'immersion.
6. Étude de l'influence de l'ordre du prétraitement combiné fongique-oxydant sur les caractéristiques de la biomasse et sur la production de biogaz.

7. Etude préliminaire de la bioaugmentation avec une nouvelle souche de champignon anaérobie, *Feramyces austinii*, pour étudier son effet sur la production de biogaz à partir de la paille.

Ces objectifs sont présentés dans le manuscrit de la manière suivante :

Dans le chapitre III de cette thèse, une revue de la bibliographie est présentée sous la forme d'une collection de trois articles publiés. Le premier article publié dans *Bioresource Technology* en 2021 traite de l'efficacité de différents prétraitements biologiques et chimiques/physicochimiques combinés sur la dégradation des fibres et la production de sucre. Il a été établi qu'il est important de déterminer l'ordre de prétraitement dans les stratégies combinées sur la base du mécanisme d'action des méthodes de prétraitement individuelles. Le second article, publié dans *Applied Microbiology* en 2022, traite des stratégies de prétraitement biologique et chimique/physicochimique combinées étudiées jusqu'à présent pour la production de biogaz et de bioéthanol. Seule la combinaison de prétraitement fongique/enzyme/alcalin avait été étudiée dans le cadre d'un traitement séquentiel biologique-chimique des LCB pour la production de biogaz jusqu'à la valorisation de cet article. La troisième partie de cette section est un extrait d'un chapitre de livre qui a été publié par Springer Nature dans le livre intitulé *Fungal Biopolymers and Biocomposites : Prospects and Avenues* in 2022. La dégradation oxydative enzymatique de la lignine par les champignons y est détaillée. Les différentes techniques analytiques utilisées pour caractériser la lignine sont également abordées en détail. Cette étude approfondie a ouvert la voie au choix d'un prétraitement combiné fongique - Fenton des LCB pour améliorer la production de biogaz.

Le chapitre IV est consacré aux études de prétraitement chimique de la paille. La première partie du chapitre traite de l'optimisation du prétraitement Fenton sur la base de techniques de caractérisation simples. La seconde partie est présentée sous la forme d'un article publié dans *Energies* in 2023, qui traite en détail des difficultés liées à la caractérisation de la biomasse. Elle présente un large éventail de techniques analytiques de pointe utilisées pour la caractérisation de la biomasse afin de comprendre l'action du prétraitement physique et chimique sur la production de biogaz.

Le chapitre V traite du prétraitement biologique de la paille. Dans la première partie de ce chapitre, le potentiel du substrat de champignon usagé pour la production de biogaz est élucidé en vue de développer une bioraffinerie intégrée pour l'alimentation et l'énergie. La deuxième partie examine la variation de l'efficacité de la dégradation des fibres et de la production d'hydrates de carbone avec différentes souches de *Pleurotus ostreatus* dans des conditions d'immersion.

Le chapitre VI présente le prétraitement biologique et chimique combiné avec 2 souches de *Pleurotus ostreatus* (hybride et pure) et la réaction de Fenton pour la production de biogaz. L'influence de l'ordre du prétraitement séquentiel sur les caractéristiques de la biomasse et la production de biogaz est comparée au prétraitement fongique unique.

Le chapitre VII présente une étude préliminaire de l'utilisation de champignons anaérobies pour améliorer le rendement du biométhane. Une nouvelle méthodologie de bioaugmentation avec des champignons anaérobies filamenteux, *Feramyces austinii*, est expérimentée et fournit des perspectives sur les défis rencontrés dans la mise à l'échelle du processus de production de champignons anaérobies.

Tous les matériaux et méthodes liés à chaque objectif de recherche sont présentés dans les chapitres correspondants. Cette thèse se termine par un résumé (chapitre VIII) de tous les résultats obtenus, énonce les limites de l'étude et postule le champ d'application des recherches futures.

### **IX.3 Conclusions générales et perspectives**

Les travaux ont été menés dans le but de mieux comprendre l'efficacité du prétraitement combiné de la paille pour la production de biogaz. Dans ce contexte, les prétraitements physico-chimiques et chimico-biologiques ont été étudiés pour leur impact sur les caractéristiques de la biomasse et le potentiel de biométhane. Le prétraitement physique a été réalisé par broyage et autoclavage car il s'agit de techniques couramment utilisées en combinaison avec d'autres méthodes de prétraitement. Pour le prétraitement chimique, le prétraitement Fenton, qui est un processus de dégradation non sélective des composés organiques, a été choisi car il imite la décomposition du bois par les champignons. Le prétraitement fongique aérobie avec *Pleurotus ostreatus* est un processus bien étudié et connu pour dégrader efficacement divers substrats. Il utilise un mécanisme enzymatique oxydatif pour métaboliser les principaux constituants de la biomasse. Comme il s'agit d'une variété de champignons largement produite, appelée pleurote, il est intéressant d'utiliser les résidus de la culture des champignons ainsi que le mycélium pour la production de biogaz. D'autre part, les champignons anaérobies qui, au cours de la dernière décennie, ont gagné en popularité grâce à leur machinerie cellulolytique, ont également été étudiés pour leur effet sur l'amélioration de la production de biogaz.

#### **Principaux résultats**

Le prétraitement Fenton a été étudié avec trois concentrations différentes de  $\text{Fe}^{2+}$  et  $\text{H}_2\text{O}_2$  pour sa capacité à réduire la nature récalcitrante de la biomasse. Il a été observé que de faibles concentrations de réactifs de Fenton ( $\text{Fe}^{2+}$  et  $\text{H}_2\text{O}_2$ ) étaient suffisamment efficaces pour

provoquer la dégradation de la lignine et réduire la cristallinité de la biomasse. Cependant, ils ne sont pas assez efficaces pour provoquer une augmentation significative du potentiel de biométhane. Le prétraitement Fenton a été étudié en combinaison avec des prétraitements physiques tels que le broyage et l'autoclavage. La réduction de la taille s'est avérée être une méthode de prétraitement puissante qui a masqué l'effet du prétraitement Fenton. D'autre part, l'autoclavage a permis d'améliorer légèrement la cinétique, mais pas le rendement global du biométhane. Lorsque leurs synergies ont été étudiées ensemble, elles ont entraîné une augmentation significative du rendement en biométhane. Cependant, le prétraitement physique est un processus énergivore et il est donc important d'étudier des alternatives peu coûteuses comme le prétraitement biologique-chimique combiné.

Le substrat de champignon usagé, qui pose un problème d'élimination à l'industrie du champignon, a été étudié en tant que source de production de biogaz. Des substrats de paille de blé ont été obtenus à différents stades de la récolte de deux souches hybrides (HK35 et Spoppo) de pleurotes. Il a été observé que la souche de champignons influençait le rendement en biogaz. La paille de blé de champignon épuisée obtenue après deux rinçages de *Pleurotus ostreatus* HK35 était le meilleur substrat pour la production de biogaz par rapport à la souche Spoppo. Pour l'industrie du champignon, il est plus avantageux de récolter deux séries de champignons en utilisant le même substrat, puis d'utiliser le substrat bien dégradé pour la production intégrée d'énergie. Par conséquent, pour compenser le coût de production élevé des champignons, la production de bioénergie à partir du substrat usagé pourrait contribuer à compenser certains coûts énergétiques.

La culture de champignons en milieu solide, comme dans le cas de la production de champignons, présente l'inconvénient d'un taux de croissance lent des champignons car les conditions environnementales des microbes ne peuvent pas être régulées. La culture submergée, en revanche, assure une meilleure homogénéité des nutriments et un meilleur transfert d'oxygène. Trois souches pures de *Pleurotus ostreatus*, à savoir MUCL 20510, MUCL 28511 et MUCL 29420 de la banque de mycologie, ont été étudiées pour leur efficacité de dégradation de la biomasse en conditions immergées. Une fois de plus, des variations entre les souches pour les capacités de biotraitement ont été observées. Les variations entre les souches d'une même espèce fongique peuvent être attribuées à leurs caractéristiques de croissance, à leurs séquences génétiques et à leurs profils enzymatiques. En outre, la capacité de dégradation d'une même souche varie en fonction des conditions de culture, telles que la culture en milieu solide ou immergé, qui influencent plusieurs paramètres tels que le temps d'incubation, le pH, l'aération, etc. Il est donc nécessaire de cribler un grand nombre d'isolats fongiques afin de choisir la bonne souche pour un processus de prétraitement particulier.



En général, le traitement fongique unique avec la souche *Pleurotus ostreatus* MUCL 29420 a permis d'améliorer le BMP, tandis que la souche *Pleurotus ostreatus* HK35 n'a pas montré d'amélioration dans des conditions d'immersion. La combinaison du prétraitement biologique et chimique s'est également avérée bénéfique, mais uniquement en fonction de la souche utilisée. La modification de l'ordre de la séquence du prétraitement combiné affecte la solubilisation de la biomasse. La différence est due au mécanisme d'action basé sur l'ordre d'application qui diffère dans le type de substances libérées après chaque étape. En général, le prétraitement chimique suivi d'un prétraitement fongique (CP+FP) n'a pas été bénéfique pour la digestion anaérobie. En effet, le prétraitement chimique a rendu les polysaccharides inaccessibles plus accessibles pour le prétraitement fongique ultérieur. Cela a conduit à l'utilisation des substances libérées pour leur croissance et, par conséquent, à un rendement plus faible en biométhane. Le prétraitement combiné des champignons suivi de la méthode chimique (FP+CP) s'est avéré bénéfique pour la souche HK35 alors qu'il n'a pas amélioré la production de biogaz pour la souche PO3 par rapport au prétraitement fongique seul. Par conséquent, l'efficacité du prétraitement biologique et chimique combiné est également spécifique à la souche. L'ordre de prétraitement est également très important car il affecte le mécanisme d'action sur la biomasse, ce qui a une incidence sur la production de biogaz.

La bioaugmentation avec des champignons anaérobies est un domaine de recherche relativement nouveau et passionnant pour améliorer la production de biogaz. La production de champignons anaérobies s'est avérée assez difficile en raison de sa maintenance élevée. *Feramyces austinii* a permis d'améliorer le taux d'hydrolyse et la production globale de méthane de la paille. La délignification de la lignocellulose par les champignons anaérobies a été observée, ce qui rend l'holocellulose plus sensible à la dégradation bactérienne dans des conditions anaérobies. D'autres études de génotypage et des études enzymatiques pourraient aider à expliquer les relations symbiotiques de *Feramyces austinii* et sa capacité à dégrader la lignine dans des conditions anaérobies.

### **Limites et perspectives**

Le prétraitement fongique est une méthode respectueuse de l'environnement et s'avère efficace pour la production de biogaz à partir de certaines souches et conditions de culture. Les différences de capacité de dégradation entre les souches d'une même espèce fongique rendent difficile le transfert de technologie à grande échelle. Par conséquent, l'analyse génomique et enzymatique de différents isolats doit être effectuée afin de mieux choisir la bonne souche pour l'étude. Néanmoins, outre la souche de champignon, les conditions de prétraitement et l'espèce de biomasse affectent également la production de biogaz. Par conséquent, une analyse

multivariée du prétraitement combiné peut aider à optimiser davantage les conditions de prétraitement. Les champignons anaérobies ouvrent un vaste champ de recherche pour leur relation synergique avec les méthanogènes et leur action enzymatique. Bien que cette recherche mette l'accent sur la nécessité d'exploiter les champignons anaérobies inexplorés pour la production de biogaz, la culture et la maintenance de ces champignons restent un énorme défi. Il est nécessaire de développer pleinement les techniques de culture pour la production à grande échelle de ces champignons avant qu'ils puissent être largement utilisés pour améliorer la production de biogaz. L'étude de la synergie entre les champignons anaérobies et les méthanogènes peut également fournir des connaissances sur le type d'inoculum à utiliser pour les protocoles de bioaugmentation. La complexité de la matrice lignocellulosique et l'hétérogénéité de la composition physicochimique de la biomasse la rendent complexe à étudier. Le résultat des différentes méthodes de prétraitement appliquées à la biomasse n'est pas suffisamment décrit par les techniques de caractérisation de pointe actuellement disponibles. Par conséquent, les études de prétraitement actuelles ne peuvent être considérées que comme des études au cas par cas. Il est donc nécessaire d'améliorer et de développer davantage les techniques analytiques afin de pouvoir comprendre les changements structuraux et chimiques que subit la biomasse au cours du prétraitement. Cela permettra de développer des modèles pour prédire le potentiel de biométhane d'une matière première. En outre, une analyse de l'énergie et des coûts des différents prétraitements doit être effectuée pour mieux estimer le niveau de préparation au transfert de technologie.



## X References

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## XI Annex

### XI.1 Chemically and physically pretreated straw in moderate conditions: Poor correlation between biogas production and commonly used biomass characterization – Supplementary Materials

Shruthi Meenakshisundaram<sup>1</sup>, Vincenzo Calcagno<sup>1,2</sup>, Claire Ceballos<sup>1</sup>, Antoine Fayeulle<sup>1</sup>, Estelle Léonard<sup>1</sup>, Virginie Herledan<sup>2</sup>, Jean-Marc Krafft<sup>2</sup>, Yannick Millot<sup>2</sup>, Xiaojun Liu<sup>1</sup>, Claude Jolivalt<sup>2, \*</sup>, André Pauss<sup>1, \*</sup>

<sup>1</sup>Université de technologie de Compiègne, ESCOM, TIMR (Integrated Transformations of Renewable Matter), Centre de recherche Royallieu - CS 60 319, F-60 203 Compiègne Cedex, France

<sup>2</sup>Sorbonne Université, CNRS, Laboratoire de Réactivité de Surface (LRS), 4 place Jussieu, F-75005 Paris, France

\* Correspondence: CJ (claude.jolivalt@sorbonne-universite.fr); AP (andre.pauss@utc.fr)

#### 1. Second derivative analysis of Infrared spectra

Second derivative analysis was calculated after Z-score normalization of the spectra and by applying the Savitzky and Golay algorithm (21 smoothing points, 2<sup>nd</sup> order polynomial fitting).

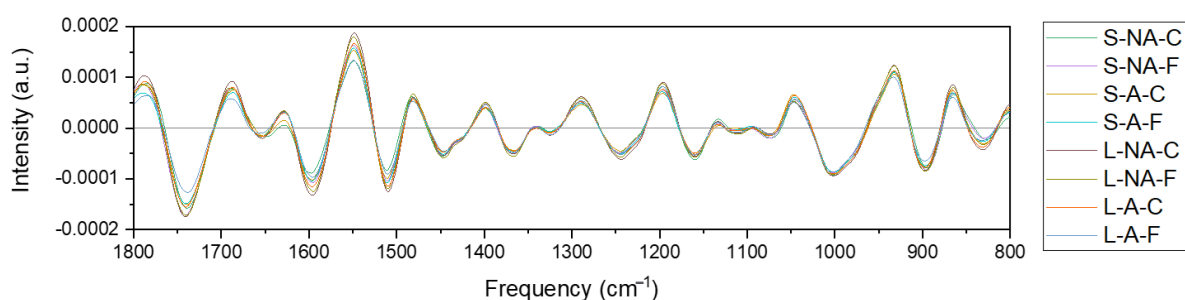


Figure SXI-1: Second derivative spectra from DRIFT spectra of pretreated samples and controls.

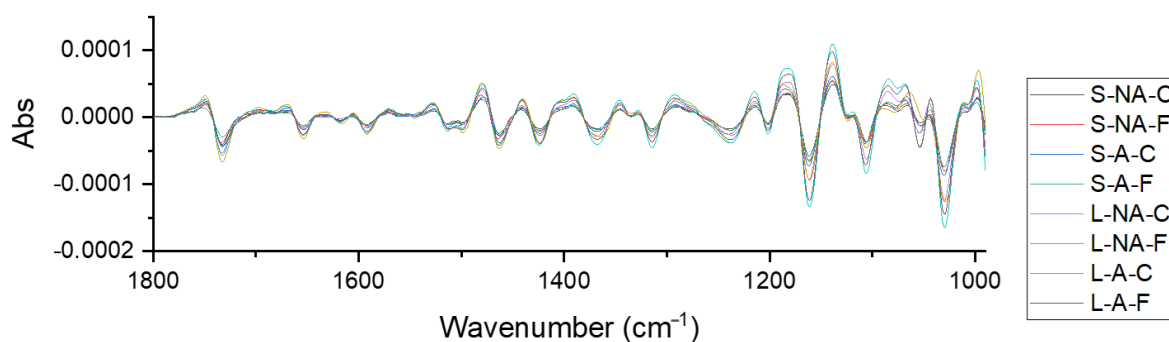


Figure SXI-2: Second derivative spectra from ATR spectra of pretreated samples and controls

## 2. Lignin isolation and digital subtraction from NMR spectra.

Cellulose was isolated by applying a two steps procedure proposed by Bernardinelli et al. (2015) (Bernardinelli et al., 2015b; Rezende et al., 2011b) with some modifications. Briefly, in the first step straw was hydrolysed with a diluted  $\text{H}_2\text{SO}_4$  solution (1% v/v in water) for 40 min at 120 °C, where a 1:20 solid to liquid ratio (g sample/ ml solution) was used. The solid mass was then separated from the hydrolysate by filtration and washed thoroughly with distilled water. The hydrolysate solution was collected, neutralized, dried with a rotavapor, and solubilized in ethanol to remove the salts. Finally, it was freeze-dried to obtain a solid yellow. In the second step, NaOH solution (4.0% w/v) at 120 °C for 40 min was used to dissolve lignin. A 1:20 solid to liquid ratio (g sample/ ml solution) was used. The solid mass was then separated from the hydrolysate by filtration, washed thoroughly with distilled water and dried in oven at 60 °C for at least 24 h. The hydrolysate solution was collected and acidified with HCl 3M to pH 2, thus observing the formation of a solid suspension. The suspension was centrifuged (2000 rpm, 5 min) three times, the supernatant removed, and finally dried in oven at 60 °C for at least 24 h. The obtain solid thus contains only lignin.

Lignin spectra was then acquired with multi-CP MAS  $^{13}\text{C}$  NMR and used for the digital subtraction, before performing the spectral fitting.

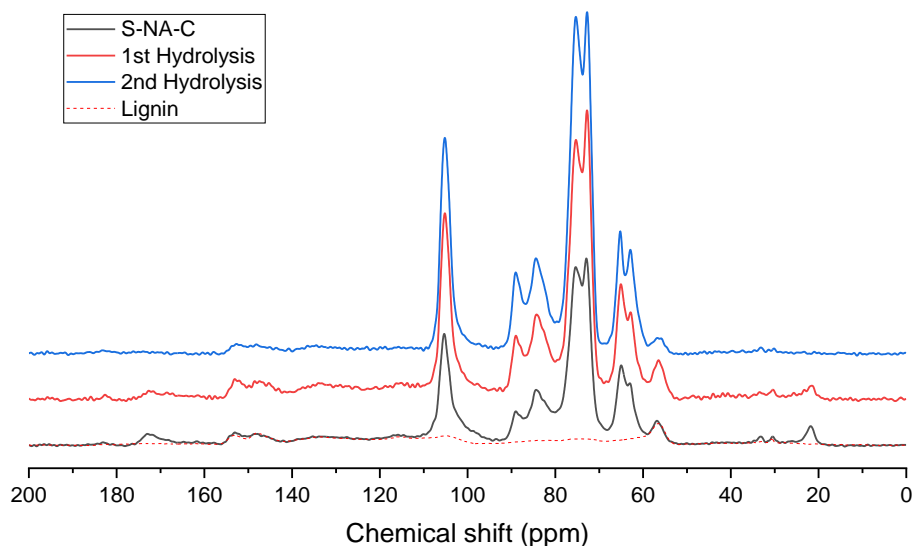


Figure SXI-3: Multi-CP spectrum of S-NA-C sample (black line) and after the first (red line) and second treatment (blue line). The spectra are normalized with respect to the maximum peak at 73 ppm. The dotted lines represent the scaled Multi-CP spectrum of the precipitated supernatant (lignin) resulting from the second treatment.

### 3. Spectral fitting of NMR spectra

All sample have been analysed by multi-CP MAS  $^{13}\text{C}$  NMR method (Figure SXI-4).

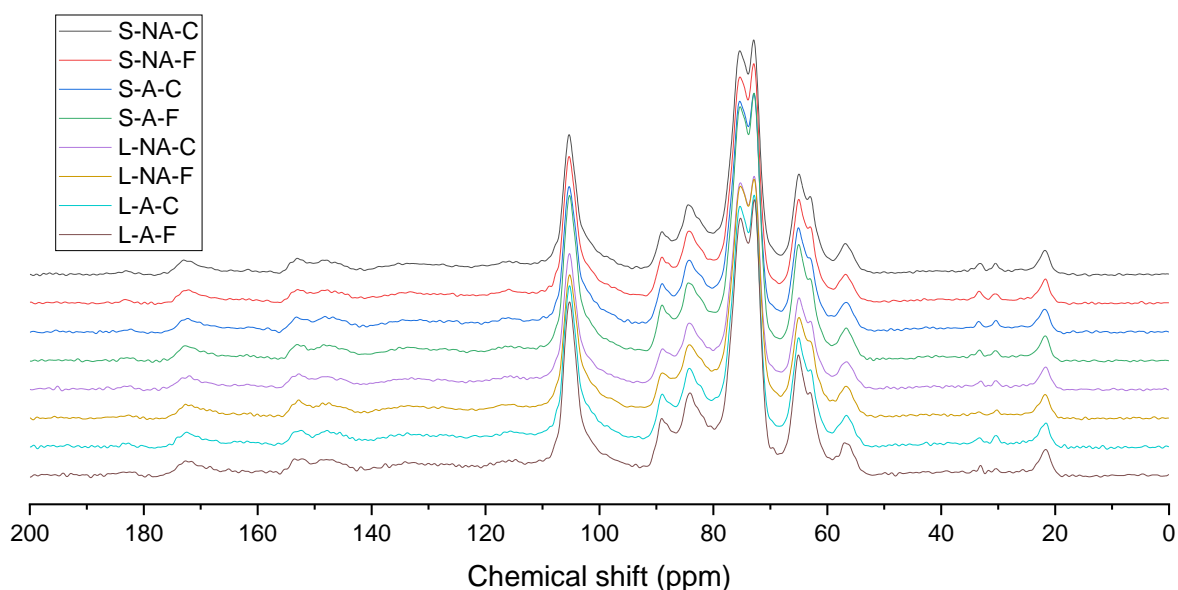


Figure SXI-4:  $^{13}\text{C}$  multi-CP MAS NMR spectra of the samples. Intensity has been scaled in dependence of the weight of each sample.

By analyzing the cellulose region of the spectra and by comparing the peaks intensity among samples, it appears clear that the absolute intensity of the peaks slightly changes from a sample to another. This is probably due to the different water content of the samples. Anyway, ones the spectra are normalized with respect to the peak at highest intensity (73 ppm, Figure SXI-5), spectra show the same peaks intensity, thus confirming that multi-CP MAS  $^{13}\text{C}$  NMR provides quantitative results. This evidence demonstrates that any change in the relative intensity of the peaks is only due to the compositional variations of the sample and the reliability of the consequent fitting.

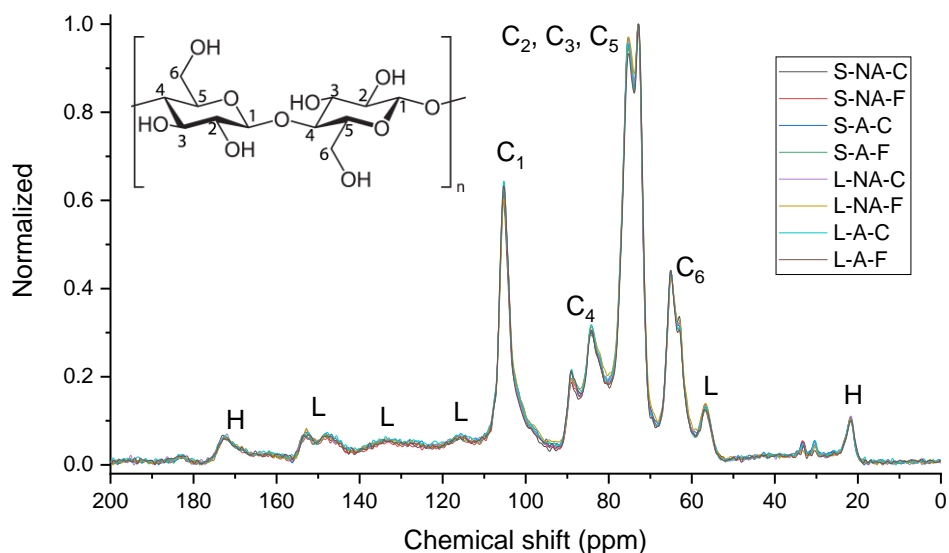


Figure SXI-5:  $^{13}\text{C}$  multi-CP MAS NMR spectra of the samples normalized with respect to the peak at maximum intensity (73 ppm, 0-1 Normalization). The spectra show the assignment peaks to the carbon in a glucopyranose repeat unit (glucose, C 1-6), to the hemicellulose (H), and to the lignin (L).

For the spectral fitting the method reported from Larsson et al. was applied (Larsson et al., 1997b). This method is based on a model for the spectral  $\text{C}_4$ -region consisting of seven distinct lines. The model includes the use of Lorentzian lines for the three signals from cellulose crystalline allomorphs  $\text{I}\alpha$  ( $\delta$  approximately at 90 ppm), cellulose crystalline allomorphs  $\text{I}\alpha+\beta$  ( $\delta$  approximately at 89 ppm), cellulose crystalline allomorphs  $\text{I}\beta$  ( $\delta$  approximately at 86 ppm), and Gaussian lines for the remaining four signals attributed to cellulose para-crystalline ( $\delta$  approximately at 88 ppm) and to non-crystalline cellulose forms, accessible cellulose at fibril surface ( $\delta$  approximately at 80 ppm and 84.5 ppm) and inaccessible cellulose at fibril surface ( $\delta$  approximately at 82.5 ppm). The overlap from the C-2, C-3, and C-5 signals, as well as hemicellulose, was considered by modelling as a single line with only its low-field tail visible in this portion of the spectrum.

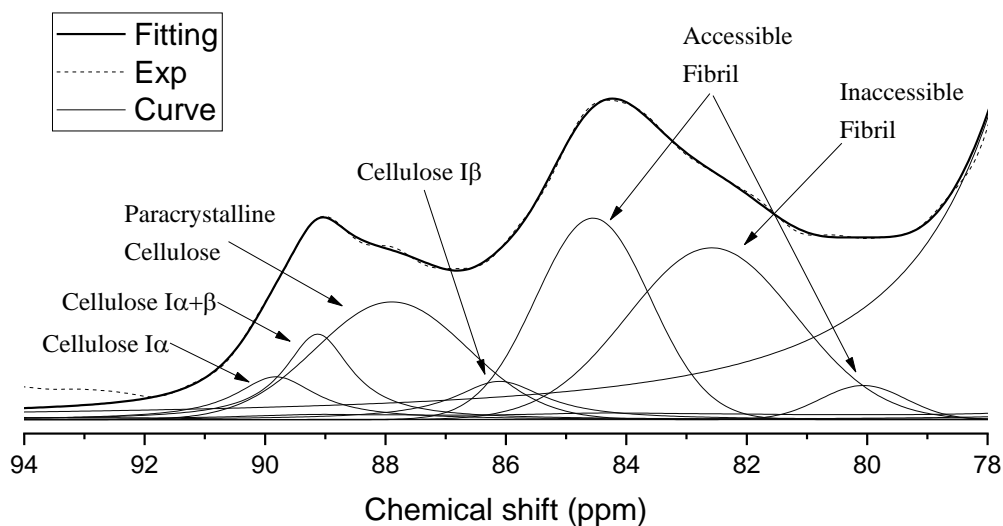


Figure SXI-6: The results of the spectral fitting of the C<sub>4</sub>-region of S-NA-C. The dotted lines represent the experimental spectrum. The fitted lines and their superposition are shown as solid lines.

**Table SXI-1:** The results of the spectral fitting of the C<sub>4</sub>-region for small and large size straws.

Assignment	Line type	Chemical shift (ppm)				FWHM (ppm)				Relative intensity (%)			
		S-NA-C	S-NA-F	S-A-C	S-A-F	S-NA-C	S-NA-F	S-A-C	S-A-F	S-NA-C	S-NA-F	S-A-C	S-A-F
I $\alpha$	Lorentzian	89.85	89.82	90.12	89.93	1.66	1.65	1.64	1.73	3.37	5.79	4.90	5.20
I( $\alpha$ + $\beta$ )	Lorentzian	89.16	89.13	89.11	89.12	1.35	1.34	1.43	1.39	9.11	9.41	12.40	11.53
Para-crystalline	Gaussian	88.10	87.89	87.94	87.93	2.98	2.97	2.95	2.95	19.10	19.44	17.87	18.88
I $\beta$	Lorentzian	86.22	86.10	86.29	86.29	1.72	1.83	1.73	1.89	7.95	5.76	5.86	6.55
Accessible fibril	Gaussian	84.57	84.55	84.56	84.54	2.17	2.23	2.24	2.27	24.04	25.02	24.15	23.52
Inaccessible fibril	Gaussian	82.63	82.58	82.49	82.48	3.39	3.29	3.41	3.35	33.79	31.41	32.41	31.57
Accessible fibril	Gaussian	79.96	80.07	79.76	79.93	1.65	1.67	1.63	1.61	2.65	3.17	2.40	2.75

Assignment	Line type	Chemical shift (ppm)				FWHM (ppm)				Relative intensity (%)			
		L-NA-C	L-NA-F	L-A-C	L-A-F	L-NA-C	L-NA-F	L-A-C	L-A-F	L-NA-C	L-NA-F	L-A-C	L-A-F
I $\alpha$	Lorentzian	89.99	89.94	89.87	90.14	1.61	1.66	1.64	1.56	5.54	6.27	5.73	2.91



I( $\alpha+\beta$ )	Lorentzian	89.06	89.04	89.09	89.24	1.35	1.34	1.34	1.34	9.20	9.14	11.74	9.06
Para-crystalline	Gaussian	87.82	87.79	87.75	88.26	3.07	3.01	2.93	2.86	19.71	19.46	19.68	21.84
I $\beta$	Lorentzian	86.03	86.06	86.10	86.33	1.83	1.76	1.76	1.83	7.39	6.23	3.61	9.46
Accessible fibril	Gaussian	84.41	84.42	84.50	84.51	2.21	2.28	2.29	2.11	24.77	26.22	24.59	21.91
Inaccessible fibril	Gaussian	82.43	82.40	82.52	82.61	3.35	3.38	3.48	3.34	30.71	30.43	32.21	32.18
Accessible fibril	Gaussian	79.88	79.89	79.77	80.08	1.69	1.60	1.53	1.66	2.68	2.25	2.45	2.63

#### 4. Spectral fitting of WAXD

The methods used was the method reported by Ahvenainen et al. and indicated as “Method 2: Gaussian peak fitting without a linear background (Gaussian peaks)” (Ahvenainen et al., 2016b). In this method, a relatively small  $2\theta$  range between  $13^\circ$  and  $25^\circ$  is used and four cellulose diffraction peaks, (110,  $1\bar{1}0$ , 102, and 200) are fitted. A fifth Gaussian is fitted as the amorphous contribution.

A linear baseline was used, whose slope and intercept were determined by using the complete profile analysis acquired ( $2\theta$  range between  $10^\circ$  and  $50^\circ$ ) (Murthy and Minor, 1990b). Peak position was determined by using the value obtained from a commercial sample of cellulose with higher crystallinity, except for the 200-diffraction peak, which is fitted to the right of the observed 200-peak maximum.

The area of the four crystalline peaks ( $A_{Cr}$ ) and the total area (crystalline and amorphous peaks,  $A_{sample}$ ) are used to calculate the crystallinity as shown below (Ahvenainen et al., 2016b):

$$CI = \frac{A_{Cr}}{A_{sample}}$$

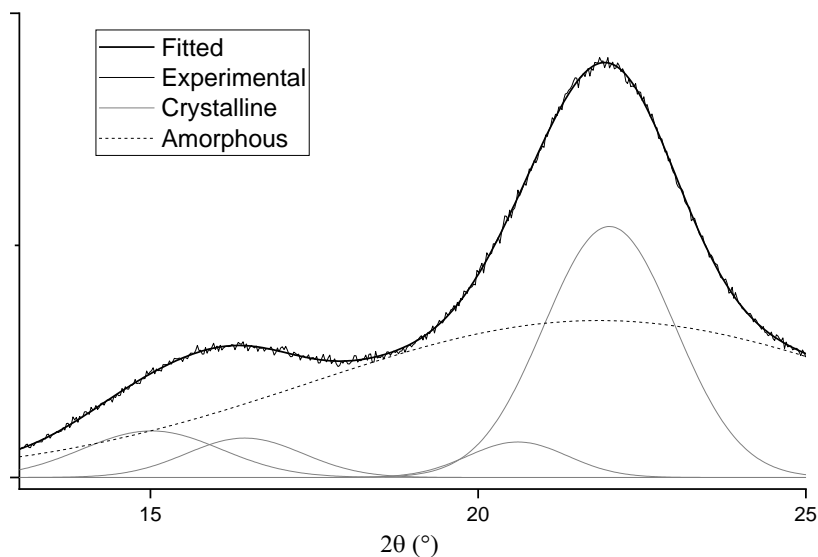


Figure SXI-7: Results from spectral fitting of the WAXD diffractograms for S-NA-C. The dotted line represents the amorphous gaussian curve. The experimental line, the fitted lines, and their superposition are shown as solid lines.

**Table SXI-2:** Crystallinity Index and Crystallite size obtained from spectral fitting of the WAXD diffractograms.

Sample name	Crystallinity Index (%)	Crystallite Size (Å)
S-NA-C	43.4	37.8
S-NA-F	49.7	38.0
S-A-C	47.6	38.5
S-A-F	46.7	39.3
L-NA-C	52.8	43.8
L-NA-F	57.5	36.4
L-A-C	51.8	37.1
L-A-F	53.8	37.5

## 5. Additional images from SEM

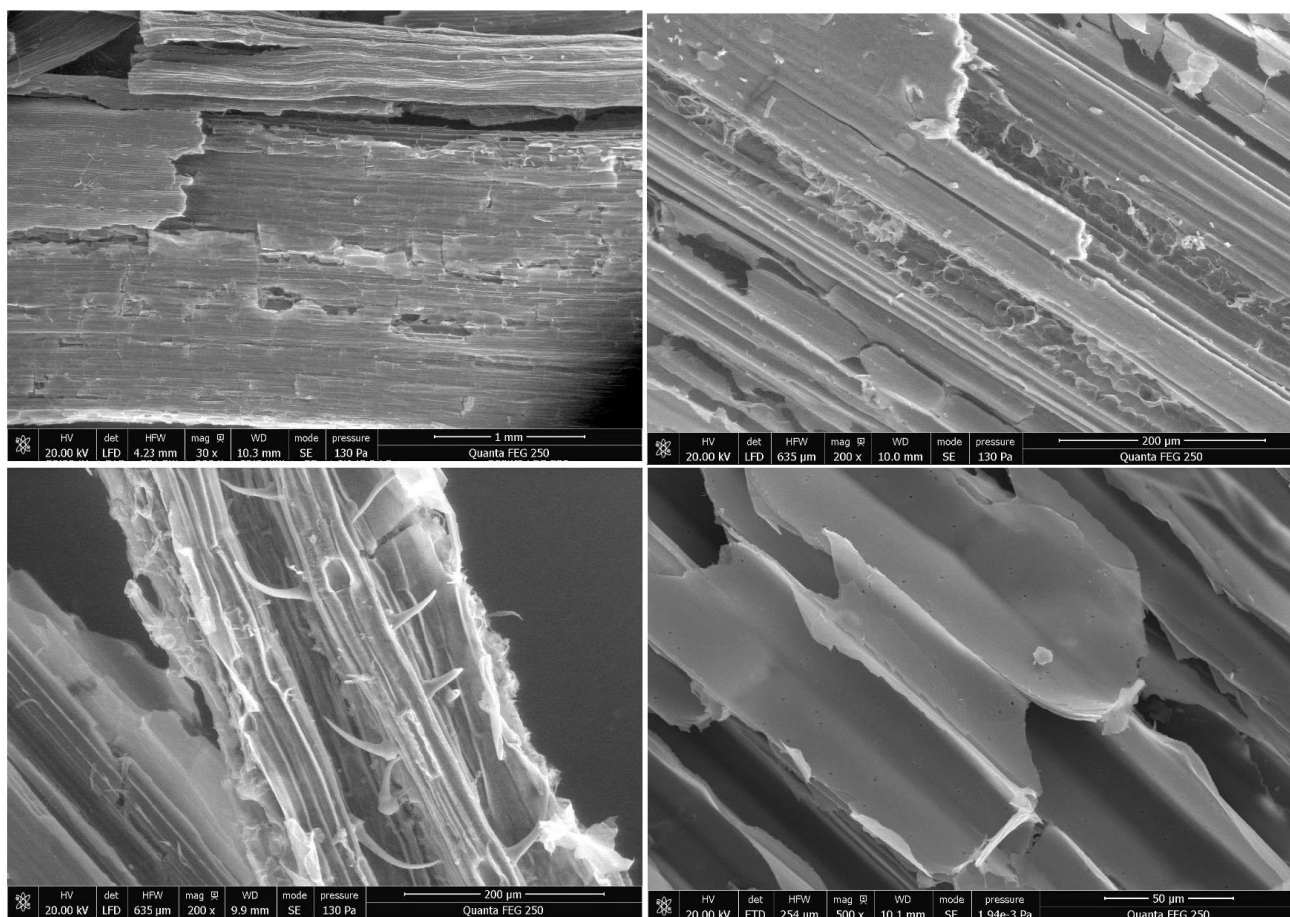


Figure SXI-8: SEM images of S-NA-C taken at different magnifications.

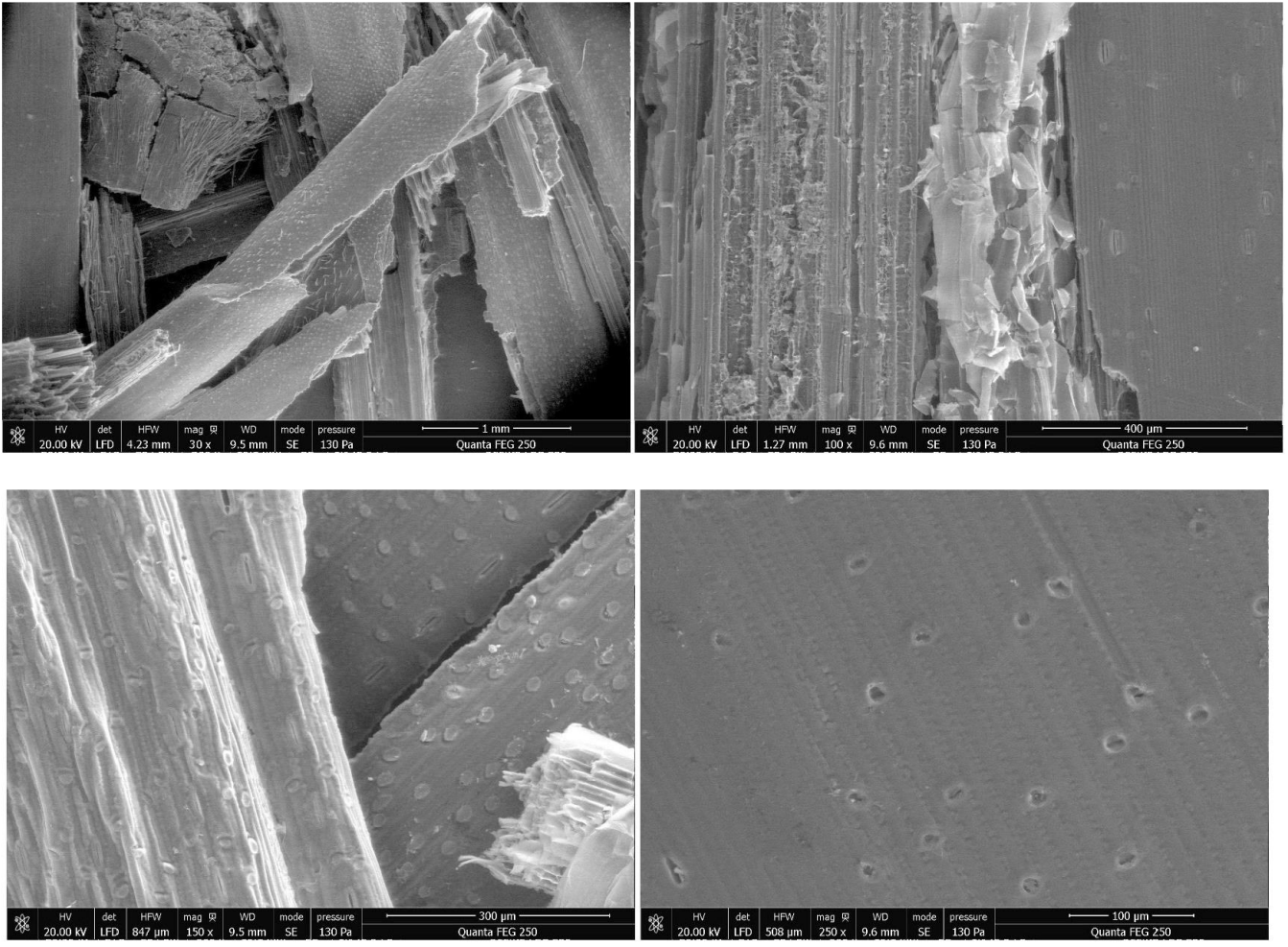


Figure SXI-9: SEM images of S-NA-F taken at different magnifications.

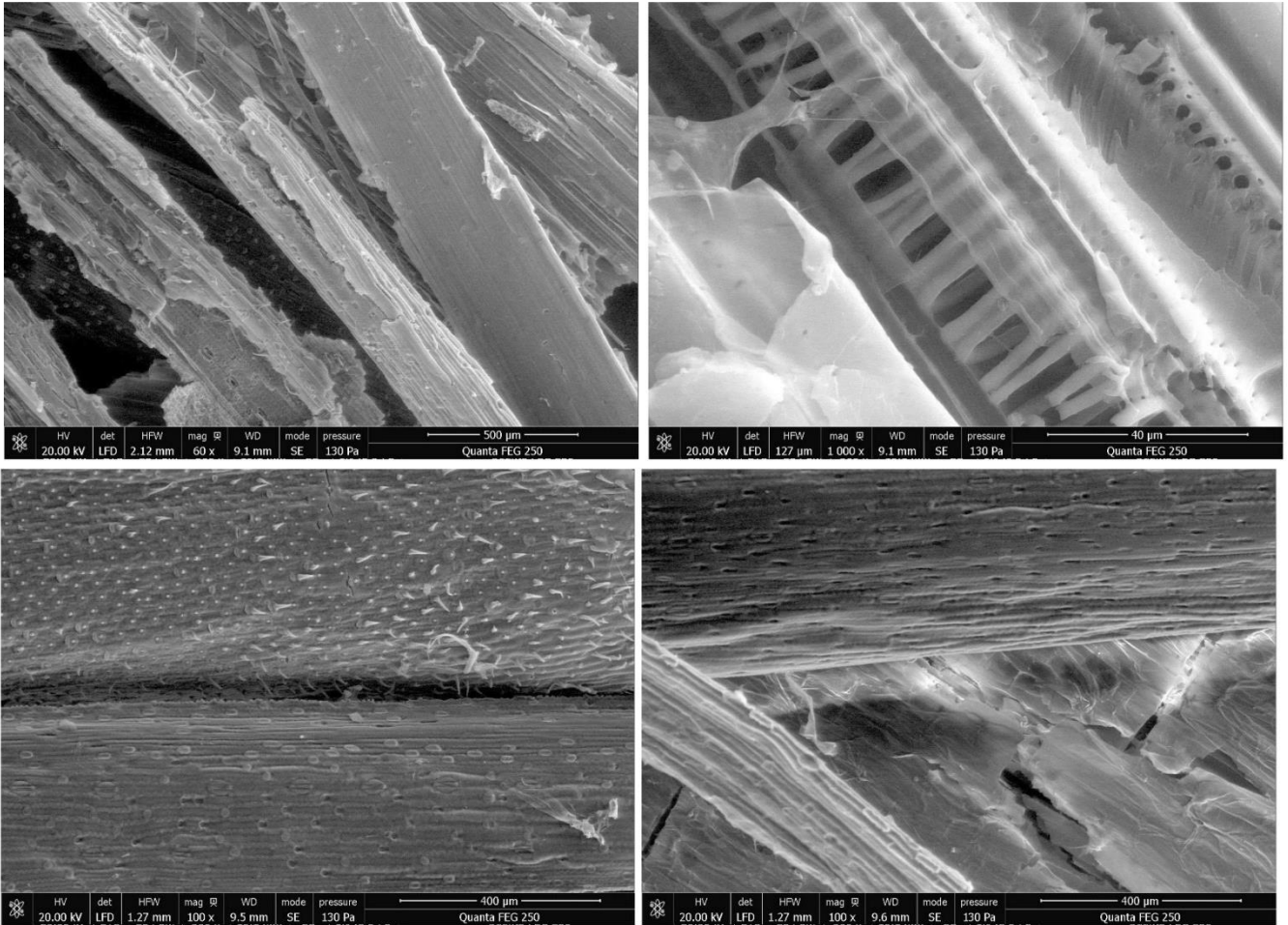


Figure SXI-10: SEM images of S-A-C taken at different magnifications.

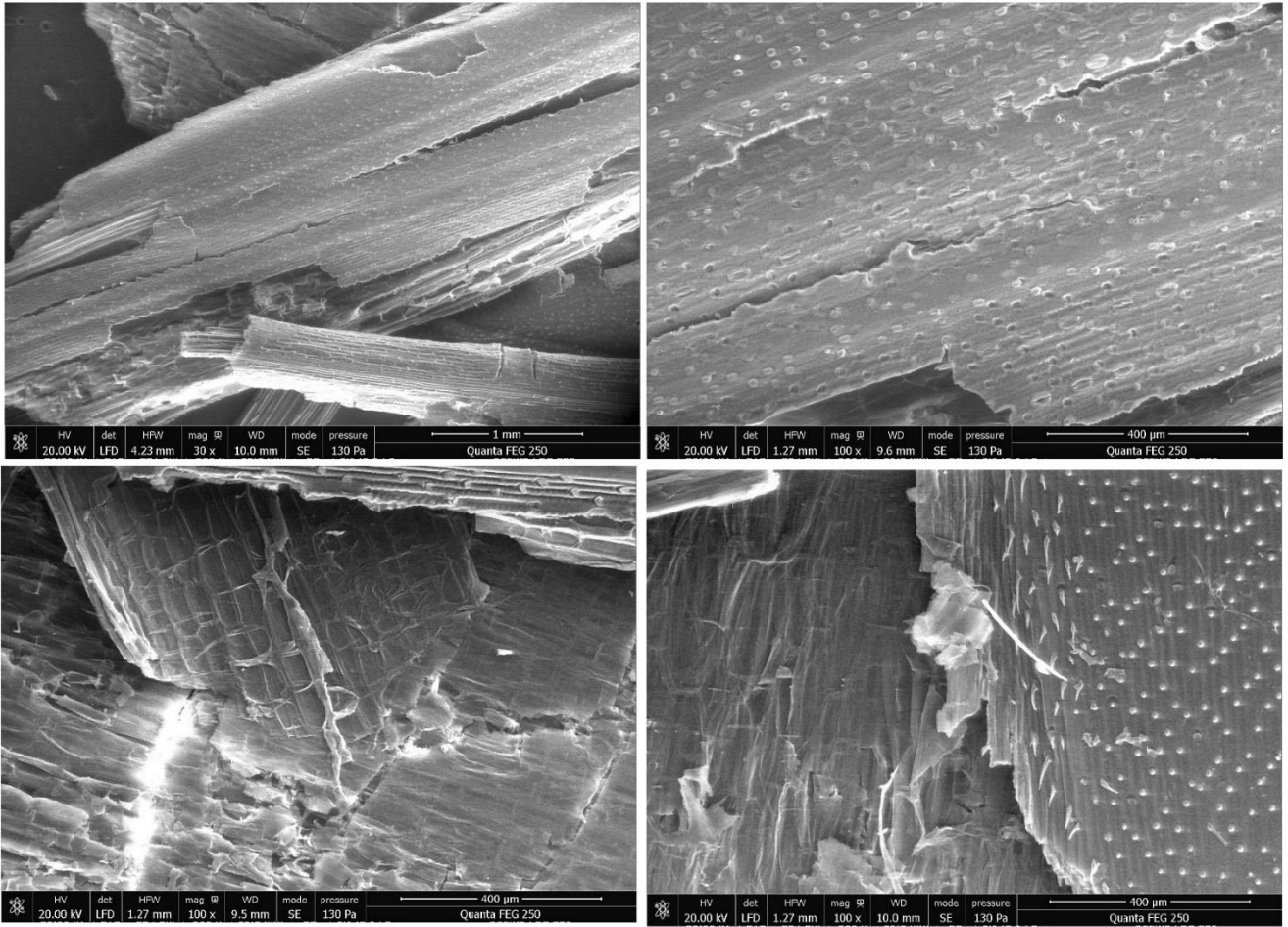


Figure SXI-11: SEM images of S-A-F taken at different magnifications.

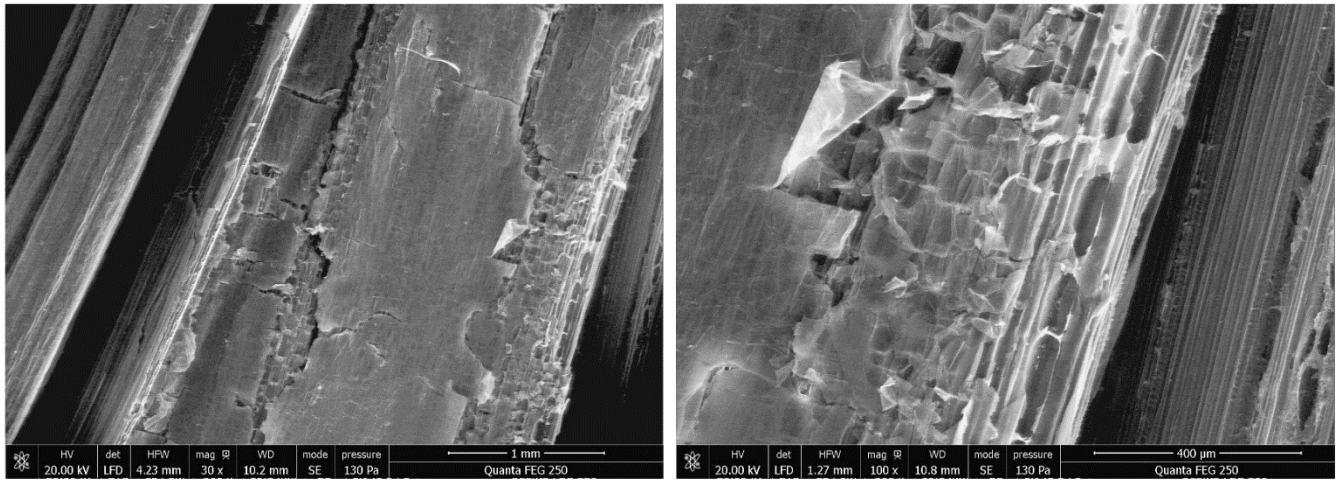


Figure SXI-12: SEM images of L-NA-C taken at different magnifications.

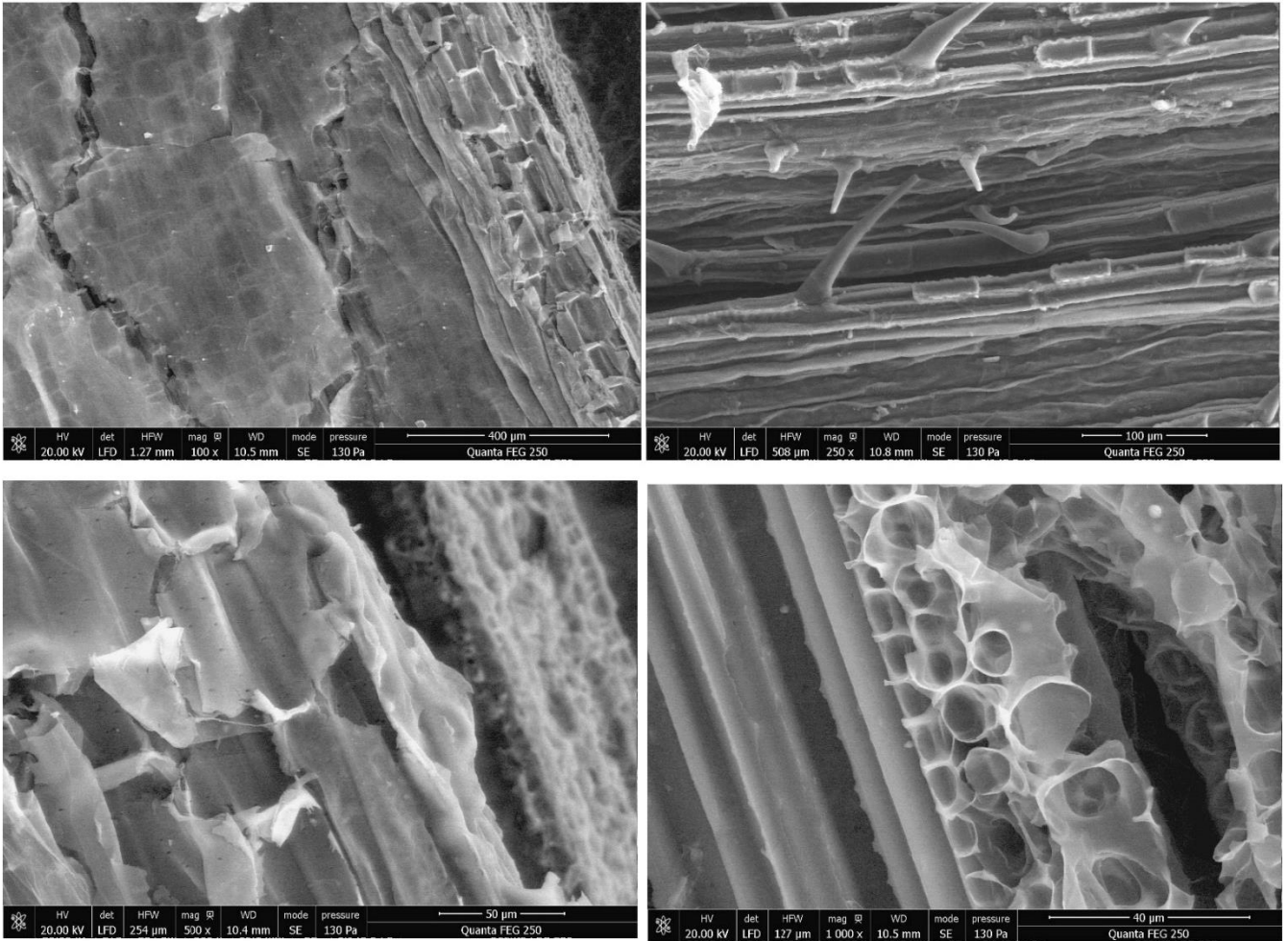


Figure SXI-13: SEM images of L-NA-F taken at different magnifications.

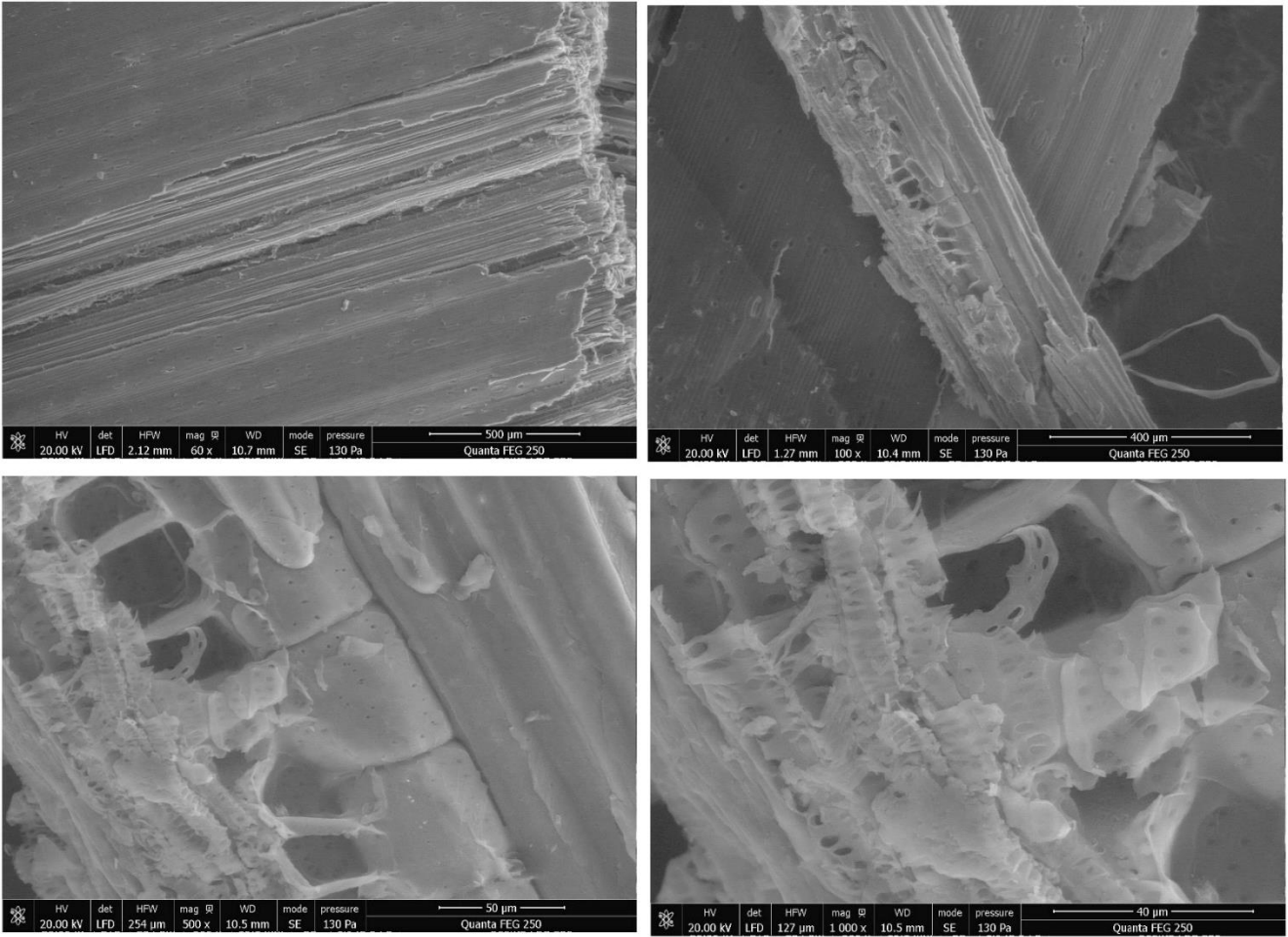


Figure SXI-14: SEM images of L-A-C taken at different magnifications.



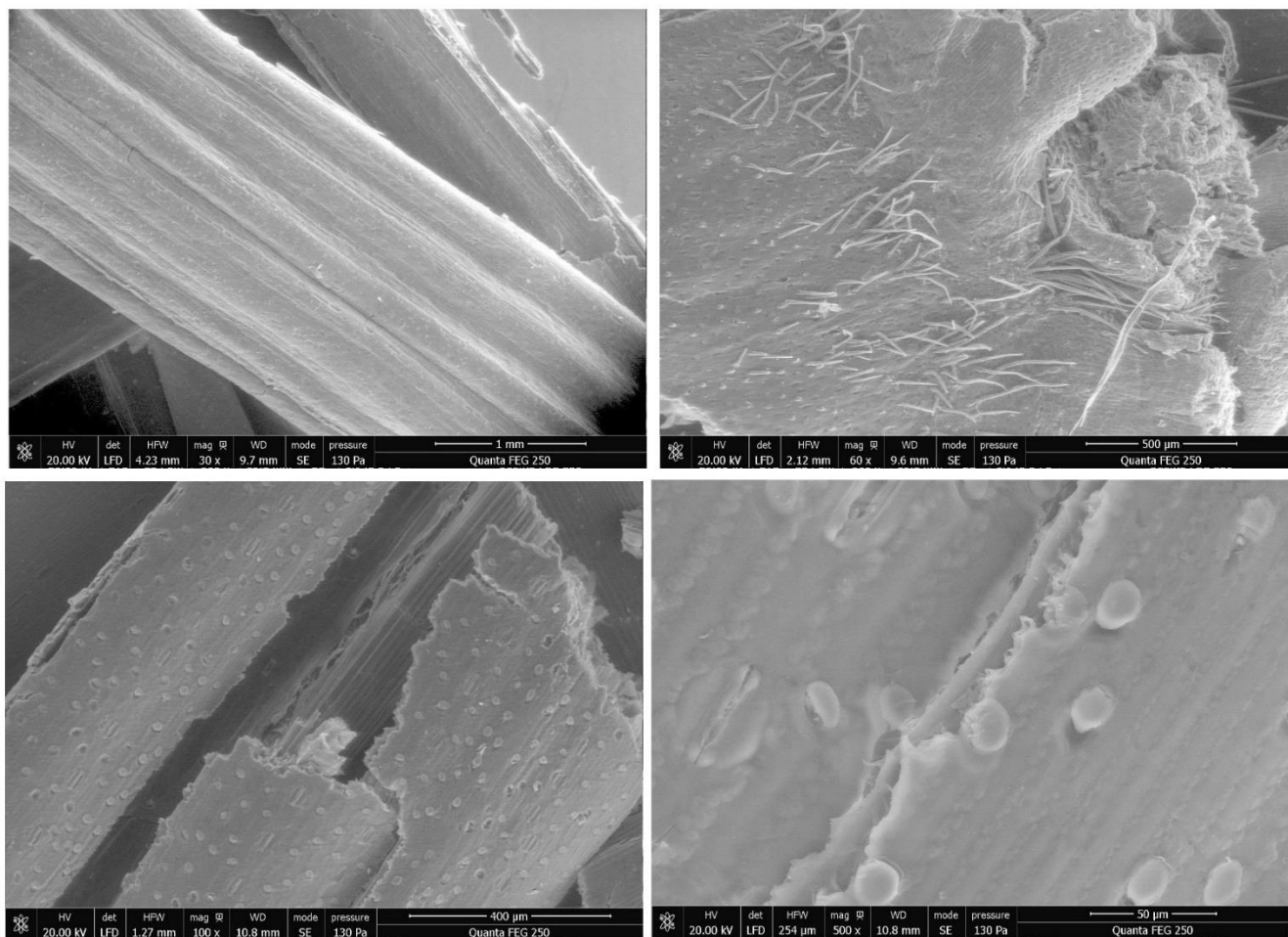


Figure SXI-15: SEM images of L-A-F taken at different magnifications.

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