Study of the valorization of pretreated hemp inflorescence or shive for the production of fungal enzyme by solid-state fermentation

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Diplôme : Master en bioingénieur : chimie et bioindustries, à finalité spécialisée
Année académique : 2016-2017
URI/URL : http://hdl.handle.net/2268.2/3042

LIÈGE université
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[1]Dans ce cas, l'autorité académique est représentée par le promoteur membre du personnel enseignant de GxABT.

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ABSTRACT

This study was performed at the University of Liège in the faculty of Gembloux Agro-Bio Tech with the collaboration of the Laboratory of Biomass and Green Technology and the Laboratory of Bio-industry.

English

Background The independence toward fossil-fuel is one of the biggest challenges of the 21st century. To deal with the issue, some people chose to get back in time and bring back to light the cultivation of industrial hemp. As the interest of the public is growing, the number of products use of the plant is constantly increasing, reaching new markets. Despite the growing hemp movement emerging, it is crucial to find new ways to valorize the whole plant in order to support the industry. It is essential to diversify the applications where hemp could be implemented as a key stone for the bio-industry sector. It is from this perspective that this work is investigating the potential valorization of by-products of the hemp industry (shives and flowers) by using them as substrate for the production of enzymes. Shives and inflorescences were pretreated by steam explosion at 10 and 20 bar (~200 and 215°C) to increase the accessibility of the biomass and extract the secondary metabolites from the flowers. Afterward the solid fraction was used as substrate for the production of two enzymes (β-glucosidase and xylanase) in solid-state fermentation by two different fungi (*Sarocladium kiliense* and *Penicillium canescens*).

Results The pretreatment by steam explosion revealed itself to be beneficial to the enzymes yields. Experiments highlighted a lack of nitrogen in the shives (unbalanced C:N ratio of 140:1) and a poor proportion of polysaccharides in the inflorescences (19.2 ± 2.5 %), undesirable for the induction of enzymes. The supplementation of nitrogen showed a huge increase in xylanase productions with a maximum of 2595 ± 280 U/gds on pretreated shives (20 bar) by *Penicillium*. The highest β-glucosidase productions (4.05 ± 1.03 U/gds) were collected with the same fungi growing on milled shives.

Conclusion Inflorescences have a poor recovery in the steam explosion and therefore they are less appropriate as substrate for solid-state fermentation. Hemp shives have better recovery in the steam explosion and have higher enzymes yields once the conditions of fermentation are optimized.

Keywords industrial hemp, pretreatment, steam explosion, solid-state fermentation, enzymes

Cette étude fut réalisée à l'Université de Liège dans la faculté de Gembloux Agro-Bio Tech grâce à la collaboration du Laboratoire de Biomasse et Technologie Verte ainsi que le Laboratoire de Bio-industries.

Français

Contexte L'indépendance face aux énergies fossiles semble être un des grands défis de ce 21ème siècle. Face à ce problème, certaines personnes ont choisis de retourner quelques années en arrière afin de remettre au goût du jour la culture du chanvre industriel. Cette plante redécouverte récemment voit lentement sa reconnaissance atteindre le grand public et gagner de nouveaux marchés. A ce jour en revanche, certaines parties de la plante manquent toujours d'applications concrètes et restent inutilisées.

L'objectif de ce travail est d'examiner le potentiel de valorisation des sous-produits de l'industrie du chanvre. Bien que le chanvre industriel soit de plus en plus reconnu comme une culture à haut potentiel, il est important de diversifier les applications pour valoriser l'entièreté de la plante. Ce travail s'inscrit dans cette perspective en voulant étudier le potentiel de valorisation des résidus actuels de la plante (chènevotte et inflorescences) comme substrat solide pour la production d'enzymes (β-glucosidase et xylanase). Les résidus ont préalablement été prétraités par steam explosion à 10 et 20 bar (~200 et 215°C) pour augmenter l'accessibilité au substrat et favoriser la croissance des deux champignons filamenteux étudiés (*Sarocladium kiliense* et *Penicillium canescens*).

Résultats Le prétraitement à la steam explosion s'est révélé bénéfique pour la production d'enzymes. Les expériences ont mis en évidence un manque important d'azote dans la chènevotte (ratio C :N de 140 :1 étant non équilibré) et une faible proportion de polysaccharides dans les inflorescences (19.2 ± 2.5 %), indésirable pour une bonne induction d'enzyme chez le champignon. La supplémentation en azote dans les nutriments ajoutés à la chènevotte ont grandement augmentés les rendements en xylanases (jusqu'à 2595 ± 280 U/gds) chez *Penicillium*. Les plus hauts rendements en β-glucosidases (4.05 ± 1.03 U/gds) ont étés obtenus sur de la chènevotte ayant subi un broyage prélablement à la fermentation.

Conclusion Le taux de recouvrement de la biomasse des inflorescences dans la steam explosion est très faible et ainsi moins adapté pour être utilisé comme substrat pour la fermentation solide. En revanche les rendements en biomasse pour la chènevotte sont bien meilleurs et productions en enzymes sont supérieures une fois les conditions optimisées.

Mots-clefs chanvre industriel, prétraitement, steam explosion, fermentation solide, enzyme

ACKNOWLEDGEMENTS

First of all I would like to thank Prof. Jacques and Prof. Richel for coming together with an interesting subject for my master thesis. Working with hemp has been in my mind since the start of my master degree and now the first step is done. Other steps will follow.

I would like to thank the laboratory technicians from the Laboratory of Biomass and Green Technology as well as those of the Bio-industry Laboratory who helped during the experiments. Thank you to Cathy and Cedric who shared their experiences and gave me many tips during the culture of microorganisms. Thanks to Maroua who helped in the characterization of the wheat straw.

These months of work passed so quickly thanks to my friends; Thomas who helped me to perform some experiments; Thibaut my fierceful opponent; and Quentin my *senpai* without whom the path of the last years would have been much different. I do not forget Mireille who boosted my will and motivation throughout this time.

Finally, I would not be here without the support of my family. They believed in me at every moment of this journey and if I was able to get through this long and rocky road, it is thanks to them. I cannot be grateful enough.

LIST OF ABBREVIATIONS

STEX= Steam explosion

EIHA= European Industrial Hemp Association

SmF= Submerged fermentation

SSF= Solid-state fermentation

CBD= Cannabidiol

THC= Tetrahydrocannabidinol

NREL= National Renewable Energy Laboratory

InU= Inflorescences without pretreatment (untreated)

In10= Inflorescences pretreated by steam explosion at 10 bar

In20= Inflorescences pretreated by steam explosion at 20 bar

ShU= Hemp shives without pretreatment (untreated)

Sh10= Hemp shives pretreated by steam explosion at 10 bar

Sh20= Hemp shives pretreated by steam explosion at 20 bar

HMF= 5-hydroxymethylfurfural

BG= β-glucosidase

2-F= 2-furfural

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I. State of the art

The following state of the art is divided into three subparts. The first provides a global insight of industrial hemp and its many applications. The second goes in more details in macromolecules and structures building the hemp plant and the third concerns the technology of the solid-state fermentation.

1 Industrial Hemp

1.1 Introduction

For more than 150 years, modern civilization has become more and more dependent on the oil industry. Its consumption and production has increased according to the growing demand but since 2005, the conventional oil production has reached its peak. At the beginning of the oilfields discoveries, the EROEI (Energy Returned On Energy Invested) was about 100. In the 90s this rate had fallen to 35 and was about 12 in 2007. Rates under 10 are considered as non-profitable. The demand won't stop increasing, the production is stagnant and oil becomes harder and harder to extract from the soil. For this reason the price of the oil barrel is most likely to skyrocket in the coming years [1]. Therefore the industry needs to find new approaches to replace the actual oil-based products. Going back in time and reconsider the multiple benefits of hemp, with possible new applications to the modern industry is a way to lower this dependency on oil. [2]

Hemp is a variety of the *Cannabis sativa L.* species from the Cannabaceae family (**Figure 1**). This herbaceous crop was known to man for millennia and cultivated around the world for its fibers and seeds. The plant takes only 3 months to grow and its size can reach up to 5 meters high. There is confusion in the public opinion between industrial hemp and its morphological similar cousin the Marihuana (*Cannabis indica L.*). The main difference, beside the size of the plant is the tetrahydrocannabidinol THC content in the flowers which is about 15% (percentage on a dry basis) in Marihuana, compared to less than 0.5% in industrial hemp. [2]

***Figure 1**: A 19th century illustration of* Cannabis sativa *(Photo: Wikimedia Commons).*

Industrial hemp was mainly farmed for its primary fibers and those of the shives. From a histological point of view, three types can be distinguished:

-the woody fibers centered in the core of the stem called hemp hurds derived from the cambiale activity

-the primary fibers outside the vascular system derived from the primary meristem

-the secondary fibers derived from the cambium

Gravimetrically, cellulose is the main material of the walls of fibers. Other constituents include diverse polysaccharides such as pectin and hemicelluloses, lignin and some proteins. Proportions differ from the histological origin, the cultivar and the state of growth of the plant. [2][4]

Looking at the inflorescence of the plant, the seeds are mainly produced for animal feed or to produce hemp oil by extraction. Lipids constitute about 30% ((percentage on a dry basis), proteins around 25% and carbohydrates some 30% of the seeds. Minerals, tocopherols and terpenes are found as minor constituents. From a nutritional point of view, hemp seeds have a really good balance in amino acids and fatty acids. Considering the proteins, all 20 amino acids are found in good proportions. Estedin counts for 60% of the proteins and albumin for 30%. Hemp oil is particularly interesting to provide a well-balanced proportion of ω-6 and ω-3 (3:1). This distribution of linoleic and linolenic acid is the suitable proportion for the human body. [3][5]

Fibers and seeds are currently the main products for what farmers are cultivating hemp. However, it would be relevant to investigate the composition of flowers where potential pharmaceutical components can be extracted. The most distinctive class of compounds in the hemp flowers are cannabinoids. More than 100 phytocannabinoids have been found in the plant. Even though the psychoactive constituent of cannabis (Δ9-tetrahydrocannabinol or THC) is of great interests as a drug [11], industrial and medical sectors are looking for its non-psychoactive component called cannabidiol or CBD (**Figure 2**). The reasons are for the great potential as in the pharmaceutical industry, without having to deal with all the regulatory of cannabis. [8][12]

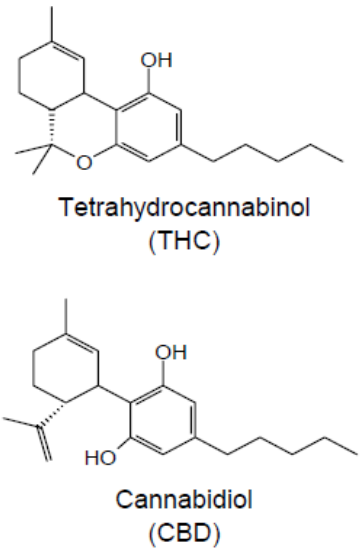

***Figure 2**: Tetrahydrocannabinol and cannabidiol molecular reprensentation. [12]*

To fully understand the coming hemp revolution, one must first look at its past. Therefore a quick summary of the story concerning the plant is necessary to understand how it went from the number one crop in the world into a complete unknown plant.

1.2 Rapid history of hemp

The hemp plant has a controversial story. For centuries, it was used in all kinds of fields for its many applications and people could recognize its usefulness at its true value. Ropes, tissues, seeds and pollen are recognizable traces of the plant to be found all around the world. The trade of hemp in Europe is believed to date back from 2000 to 1500 BC. Coming from Asia, the plant was first exported in the Middle East then in the Mediterranean Sea. [2]

Several times during history, hemp was considered as a strategic material. During the XVII and 18th century, hemp was of tremendous importance as first matter in the marine for ropes and sails. The 19th century starts to see the decline of hemp because of the technological advances of sciences. From the one hand, the modernization of the cotton industry and its development flooded the hemp market with new products. On the other hand, the emergence of the steam machine replaced hemp on boats. In the 20th century, improvement in sulfite chemistry processes (to extract the lignin) and chloride processes (for bleaching) made the wood pulp to gradually replace hemp in paper. [20]

These major technological breakthroughs were in strong competition with hemp and therefore the global hemp production decreased drastically. Numbers speak volumes: France for example was growing 175 000 ha of industrial hemp in 1830 which decreased to 12 500 ha in 1914. [2]

The war on drugs in the USA finally delivered the final blow by implementing the "Marijuana Tax Act" in 1937, making hemp no more profitable to grow. Banning the plant in the US, they quickly spread their view all around the world. The only countries resisting the ban were France and the Union of Soviet Socialist Republics. In 1992, the majority of the European countries dismissed the charges against hemp, as cultivars with really small concentration in THC emerged. [2]

1.3 The growing industrial hemp movement

Due to technological advances, new industrial discoveries and prohibition, hemp lost its place as number one crop during the 20th century. Yet the urgent need for greener and more sustainable products make the hemp plant interesting to grow once again. Industrial hemp is believed to have a global market for some 25 000 hemp-based products (F**igure 3**) in different submarkets such as food and feed, furniture, textiles, automotive, construction materials and paper. [17][18]

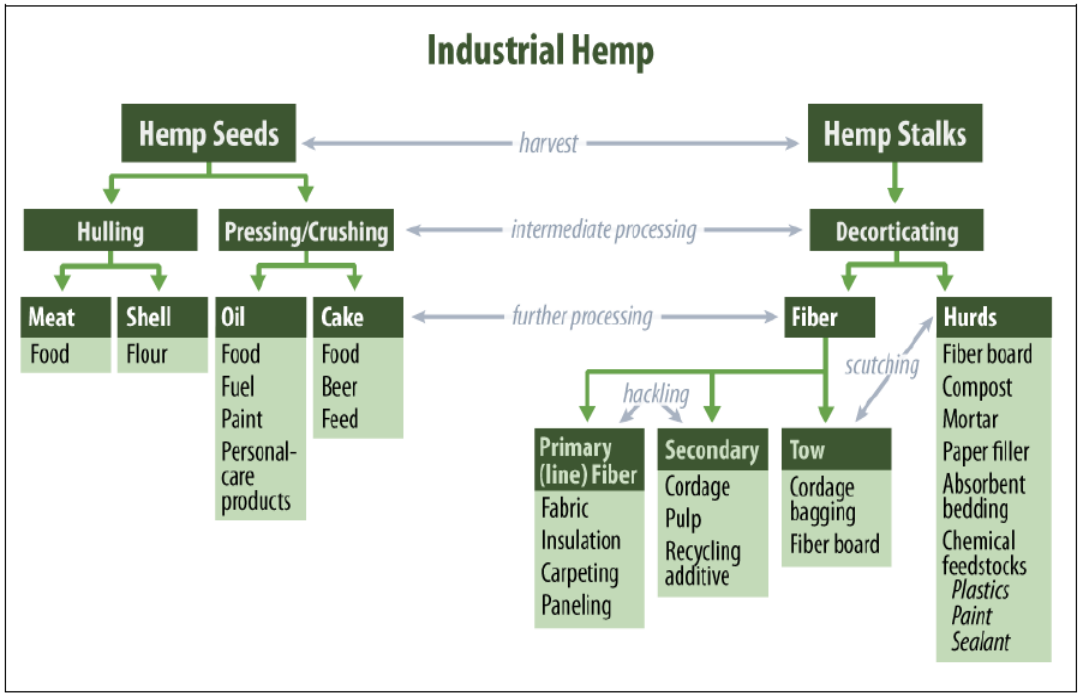

***Figure 3**: Flow chart of potential hemp-based products. Source: Congressional Research Service, adapted from D. G. Kraenzel et al., "Industrial Hemp as an Alternative Crop in North Dakota".*

Estimations from 2015 reported sales in the US at more than $580 million even though regulation in the country only started to move toward the authorization of hemp cultivation [17]. According to the European Industrial Hemp Association (EIHA), industrial hemp cultivation in Europe has twofold in the last 4 years, reaching 33300 ha in 2016.
Basic data on cultivation and processing gives a good insight of the hemp economy. Out of the 15700 ha of hemp harvested in Europe in 2013, 25000 metric tons of fiber, 43000 metric tons shives, 11500 metric tons of seeds and 240 metric tons of flowers and leaves have been processed. About 25% of the current hemp production is used for insulation. Most of current shives production goes to animal bedding and some for the construction [9]. But hemp possibilities have barely limits.

Currently, hemp has reached different industries:

- Fibers for textile, biocomposites and the pulp & paper industry:

Of all natural fibers, hemp has one of the best mechanical properties. Around 60% of hemp fibers are used for the pulp and paper industry where the main market is limited to cigarettes, bible paper and bank notes. However this share of the market is declining and new applications are coming at sight with about 25% of fibers used as insulation material and about 15% as biocomposites in automotive applications. [9][16]
Though the plant has not significantly been developed in the textile industry, there may be a bounce back. Comparing it to the actual cotton-based textile industry, hemp numbers are leading in many ways: 1.35 metric tons of cotton lint are produced per hectare where hemp produces 3 metric tons of dry fibers. The one requires an average of 9 800 liters of water for growing and processing 1 kg of cotton lint where the second only requires an average of 2 123 liters of water per kilogram of fibers, more than four times less! [10] Also, According to the Environmental Justice Foundation, 16% of global insecticide is used on the cotton-growing lands. However, hemp does not need any.

- Seeds for the food industry:

Hemp seeds are playing a significant contribution to the global hemp economy, slowly becoming an emerging food crop for the next decade. Although hemp was first produced for the fiber production, many farmers now produce the crop for seeds as sole purpose. As an

organic crop, the seed is meeting a specific market demand of people who rather pay attention to their health, preferring pesticide-free food supply. Another reason is the high nutritional value of seeds that bears proteins with high digestibility and an oil rich in omega-3 fatty acids. Its particular fatty acid composition is much appreciated by birds and fishes driving the animal feed as one of the main markets for hemp seeds. Also, antioxidant compounds such as polyphenols provide an additional nutritional value by reducing the risk of chronic diseases (cancer, neurodegenerative disorders and hypertension [6][9]). In 2016, the EIHA data showed a global European production of 11 500 metric tons of seeds compared to 6000 metric tons in 2010.

- Flowers for the cannabidiol extract industry:

Very recently, pharmaceutical and food supplement industries has increasingly been interested in cannabidiol (CBD). Numerous scientific studies show the great potential of cannabinoids. Some of them find effects on the nervous system, some as anti-inflammatory and others would suggest an anti-canceractivity. For example, El-Alfy et al.,2010 [14] found that Cannabinoids may have an antidepressant-like action and Rock et al., 2012 found that it can significantly attenuate vomiting and nausea behavior[13]. A possible anti-obesity effect is suggested as CBD has likelihood to decrease the envy of food intake. At the contrary, CBN seemed to increase feeding [15].

Hemp inflorescences contain a significant number of other compounds such as alcohols, esters, steroids, terpenes, etc… [11]. The CBD industry is currently a fast-growing market. The EIHA indicates a production for 2016 of 240 metric tons of leaves and flowers (+3200% since 2010) processed for medical applications (both THC and CBD), food supplements (CBD) and the production of essential oil. A 2 billion market is predicted for the CBD industry in Europe and North America. Although the benefits of medical cannabis are reported in many studies, countries in Europe are still very hermetic to the use and the widespread of it as a medicine. The only country with an active medical cannabis industry is the Netherlands [12].

1.4 Hemp in the age of bioeconomy

The industrial revolution of the past 150 years is showing its limits. We are now at a crucial period of transition in history where the economy needs to move away from fossil fuel toward a more sustainable path. The linear scheme of the old economy is outdated. Now, wastes should not be considered as such anymore, but as "by-product" and be reused somewhere in the industry to close the loop and become *circular*. The goal is to obtain added value from underutilized side-streams as well as reducing the amount of wastes from the industry. [30]

Implementing a circular bioeconomy is therefore what Europe is currently starting to do. For example, the article "A road map to a thriving industrial biotechnology in Europe" from the European Commission estimated the European Market of industrial biotechnology at 28 billion euro. Over half of the market was detained by antibiotics, followed by biogas and bioethanol. Thus, the EU market of biotechnology is driven by a multitude of factors like environmental issues, feedstock availability issues and regulation. The forecast for 2030 suggests that the market could reach up to 50 billion euros. [9]

As described previously, industrial hemp is a plant with a potential market of roughly 25000 products, is easy to grow in Western countries and is one of the most sustainable crop available. Hemp's high yield of natural fibers and its environmental benefits makes it a crop of value for the bio-based economy [9]. In the future, hemp may become a valuable raw material with multiple applications in the industry for a green-based and clean technology. [7]

Hemp is a growing industrial agro-resource whose composition is not fundamentally different from other agro-residues. Therefore most of its physical characteristics are related to the physico-chemistry involve in the complex lignocellulosic matrix. A description of these main features will help in understanding the plant. [4]

2 Hemp as lignocellulosic biomass

Biomass or more specifically lignocellulosic biomass refers to the plant materials mostly composed of cellulose, hemicellulose and lignin (**figure 4**). These three components gather by themselves up to 95% of the dry matter of a typical biomass. The lignocellulosic biomass composition will be detailed from an ultrastructural level (tissues, cell walls and organized crystals named microfibrils) to the supramolecular level (the crystalline formation of molecules) to the molecular level. [18]

Each cell in a plant is enclosed in an extracellular matrix called plant cell wall. The primary cell wall is thin and semi-rigid in order not to restrict the growing cells. Structural analysis reveals three classes of polysaccharides: cellulose, hemicelluloses and pectin. Once the cell's growth has stopped a more rigid secondary cell wall will be deposited to enhance the mechanical strength of the aerial structures. Lignin will be found as additional main polymer in the walls of the xylem vessels and plant fibers. The organization and composition of the plant cell walls may differ but the underlying structure remains roughly the same: strong microfibrils of cellulose linked in an amorphous matrix. While the matrix gives the resistance, the microfibril provides the needed tensile strength. [18]

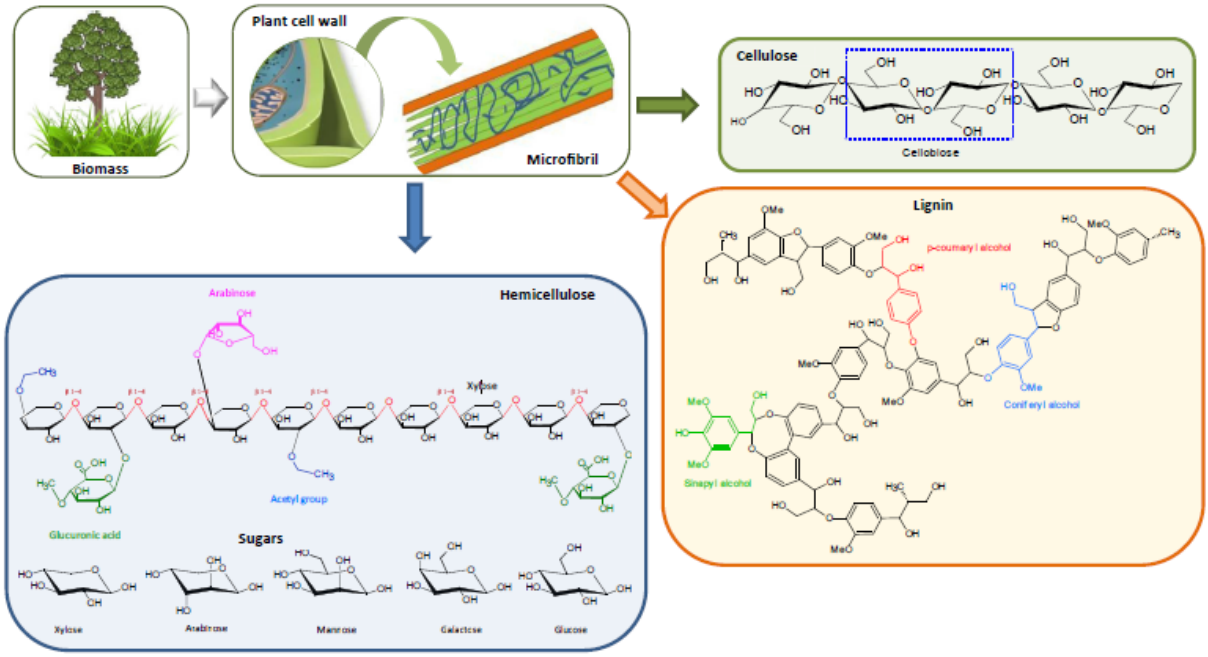

***Figure 4**: Main components and structure of the biomass: cellulose, hemicellulose and lignin [18]*

Raw hemp fiber has a different composition than hemp hurds. Fibers contain 55-72 % (percentage on a dry basis) of cellulose, 8-19% of hemicellulose and 2-5% of lignin. Hemp hurds however has a higher content of hemicellulose (31-37%) and lignin (19-21%) but a lower proportion of cellulose (36-41%). Chemical composition of the plant depends on climatic conditions, cultivar, growing technology as well as the maturity stage at harvest. [47]

2.1 Cellulose, major constituent of hemp hurds and fibers

Cellulose is the major constituent of hemp fibers and hurds. It is a linear molecular structure of β-1,4-D-glucan, polymer of -D-anhydroglucopyranose units linked by β-1,4- glucosidic bonds. Two glucose molecules counts for a molecule entitled "cellobiose" which is considered by some researchers as the building blocks of the cellulose. The crystalline cellulose fibers are possible thanks to the glucose molecules structure containing three hydroxyl groups (OH), forming intra- and inter molecular hydrogen bonds thanks to the oxygen and hydrogen atoms. The degree of polymerization (number of glucose units in the chains) is around 7000 in purified cellulose from raw hemp fibers. [21]

Both hemp hurds and fiber possess a high percentage of cellulose in their composition. The two are closely linked together in the plant. The cross section of the plant (**Figure 5**) depicts the different layers forming the complex structure in the stem. The epidermis covers the outside of the stem while bast fibers and hurds follow as inside layers. Two types of fibers are found in the hemp stem: the bast fibers composed of 5-55 mm long primary fibers and secondary fibers of 2 mm long in the hurds. They have similar diameters though the cell walls of the bast fibers are more than 5 times thicker [48].

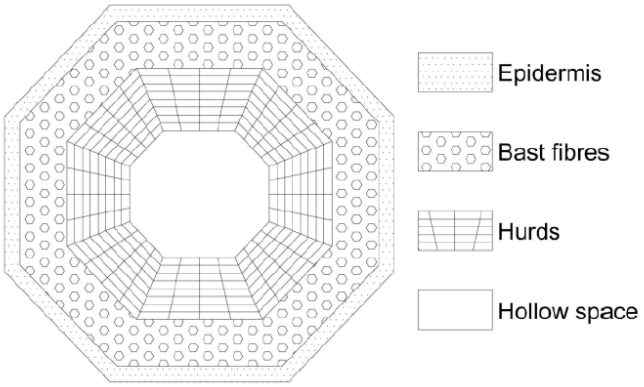

***Figure 5**: Cross-section of a hemp stem. [48]*

Hemp hurds is particularly suited for thermal insulation composites because of its good thermal properties and strength coming from the small pores inside the structure. The sizes of the pores are smaller than 400 μm and the porosity was determined to be of 57%. [47]

Hemp fibers are suited for textiles and paper thanks to their high cellulose content providing stability and strength. Physical properties of the fiber are influenced by other components in presence (hemicellulose and lignin). Thus removing those from the fiber increase the mechanical properties. The crystallinity of the cellulose plays another key role in its strength. [49]

2.2 Hemicelluloses as second major polysaccharide

Hemicelluloses are the second most abundant components present in biomass representing ~20-40% of it by weight. It is the fraction of the biomass insoluble in water but which can be extracted by alkaline treatment. Hemicellulose is a group of low molecular weight matrix polymer, associated with other polymers (mainly with cellulose) present in the plant cell walls. [18]

Also known as cross-linking glycans, the hydrogen bonding allow them to bind to the surface of the cellulose microfibrils. Hemicelluloses refer to complex heterogeneous polysaccharides of β-1,4-linked-D-pyranosyl residues in equatorial configuration and is composed of a variety of 5-carbon sugars (xylose and arabinose), 6-carbon sugars (glucose, galactose, mannose, galacturonic acid and glucuronic acid) and 7-carbon sugar (4-O-methyl glucuronic acid). The main polymer chain is composed of about 90% of D-xylose and 10% L-arabinose. [19]

Hemicellulose in hemp fibers are short and highly branched polymer of pentoses (arabinose and xylose). Other constituents may be found such as glucuronic acid and acetyl groups. Its degree of polymerization varies from 20 to 300 which is much lower than in cellulose. Hemicellulose can bind to lignin via covalent bonds by its ferulic acid and *p*-coumaric residues attached. There is also a linking effect between xylan and cellulose with hydrogen bonds which proves Hemp to be a highly imbricated lignocellulosic matrix. [50]

2.3 Lignin

The lignocellulosic biomass is recalcitrant to degradation due to an amorphous three-dimensional polymer of phenylpropanoid units called lignin. This complex phenolic polymer plays a role in the hydrophobicity, the strength and the rigidity of the secondary cell walls of the plants. Its occurrence prevails in vascular plants (woody tissues). The lignin is the only natural monomer found in nature with an aromatic backbone. It is highly resistant to enzymatic degradation and mechanical disruption. The major role is to protect plants against microbial invasions and the transport through the vascular system of the water and the nutrients. [21]

As the hemp plant is part of the Angiosperm phylum, it contains the three main monomers similar to hardwood lignin: coniferyl, *p*-coumaryl and sinapyl alcohol. These monolignols form a complex structure through oxidative coupling. **Figure 6** shows the three phenyl propanoid units forming three-dimensional polymer found in hemp. They are interconnected by different types of bonds increasing the bundle tensile strength of the fiber. [50]

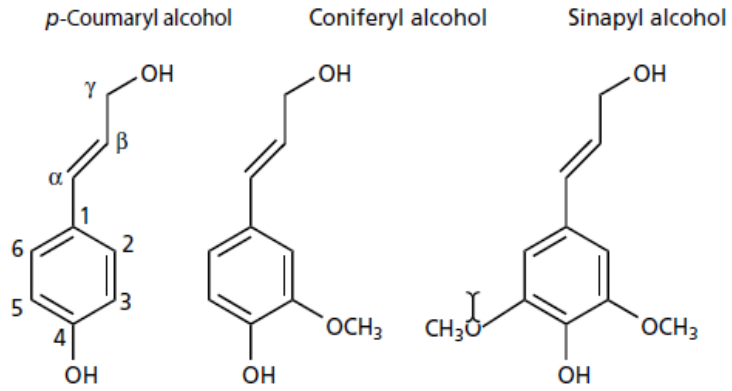

***Figure 6**: Key lignin components: p- coumaryl, coniferyl and sinapyl alcohol. [21]*

A pretreatment method is essential for the bioconversion of agricultural residues in order to get rid of the physical and chemical barriers that inhibit processes such as fermentation and hydrolysis. Out of many, the steam explosion is one pretreatment method able to overcome these limitations. [21]

Prior to biological conversion, a pretreatment step needs to be applied to overcome the recalcitrance structure of the lignocellulosic biomass. The purpose is to increase the accessibility of cellulose and hemicellulose to open the bonds with lignin for subsequent chemical conversion. There are different types of pretreatment processes, each one of them having a precise effect on cellulose, hemicellulose and lignin. The pretreatment process has a direct influence on subsequent hydrolysis, final product separation and fermentation. It has a decisive effect on fermentation toxicity, enzyme loading and enzymatic hydrolysis. Raw material characteristics and process integration will determine the choice of pretreatment required. Different kinds of pretreatments exist: Physical pretreatment, alkaline extraction, dilute acid and organosolvent are most common. A less known method combining mechanical and chemical techniques is the Steam explosion [28]. Aside from its role as pretreatment method the technique might serve as a secondary metabolite extraction method by recovering the residual liquid fraction.

3 Steam explosion as fractionation technology

The steam explosion (STEX) is a pretreatment process in which biomass is heated with high-pressure saturated steam in a reactor at a ranging temperature between 150°C and 250°C. Residence time of the steam can vary from a few seconds to several minutes before the instantaneous pressure relieve. The quick decompression is causing a mechanical disruption of the lignocellulosic matrix as the condensed moisture within the fibers evaporates and the material is recovered in the explosion tank (**Figure 7**). [23]

Steam explosion is a successful method of fractionation of the lignocellulosic biomass. The objective of the pretreatment is to alter the highly structured cellulose crystallinity by splitting the lignocellulosic fibers, solubilizing the hemicellulosic sugars and altering the lignin. Therefore the substrate will be more accessible and the enzymatic hydrolysis enhanced. The pretreated biomass is called "slurry". It is composed of the prehydrolysate (the liquid fraction) containing the main part of the degradation products generated and the solubilized sugars. The solid fraction recovers the remaining cellulose, hemicellulose and lignin. [27]

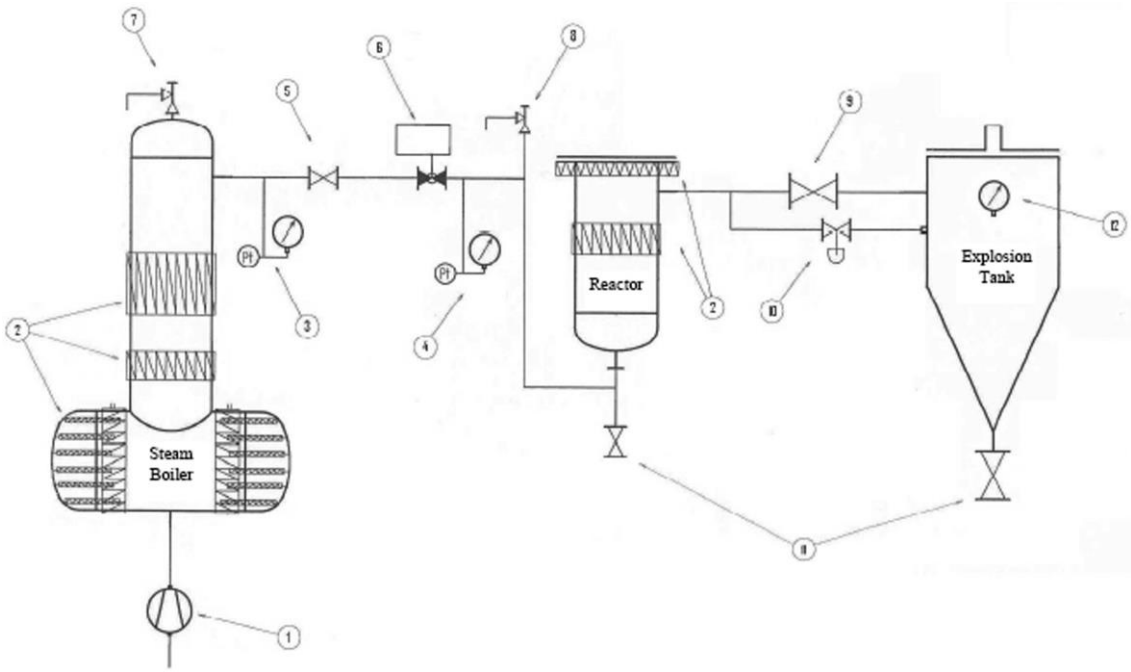

***Figure 7**: Steam explosion pilot plan at Gembloux Agro-Bio Tech. [23]*

Water at high temperature act as an acid and induces a chemical autohydrolysis in the lignocellulosic substrate. In these conditions organic acids like acetic and uronic acids are generated. Other degradation products such as 2-furfural (2-F) and 5-hydroxymethylfurfural (5-HMF) can further react and generate formic and levulinic acids which enhance the efficiency of the pretreatment. Different variables are affecting the STEX pretreatment such as size of particles and moisture of the feedstock. The importance lies in the heat transfer of the biomass. Small particles will be heated quickly and uniformly in the reactor where large size particles may overcook in the outside and be inefficiently hydrolyzed inside. [23]
Temperature and Residence time are two other essential interrelated parameters, and their performance can be estimated through a single parameter called severity factor [R_0]:

$$R_0 = t \times e^{[(Tr-100)/14.75]}$$

Where t is the residence time in minutes and Tr is the temperature of the reaction in °C.

The severity factor is useful to compare pretreatments with different range of conditions and also correlate with the recovery yield of solid and liquid fractions. [26]
Among the assets of the STEX we can count on its low environmental impact due to the absence of hazardous solvent in the process, consume less energy and other mechanical pretreatment and needs a lower capital investment. Another interest in the device is the possible use of large size chips of all different kinds of raw material and a possible scale-up to an industrial application. [25]

In order to get the most value out of the byproducts of the agro-industry, the best way is to diversify the possible outcomes. The microbial biotechnology industry is a great applicant for agro-residues used as cheap carbon sources.

4 Microbial biotechnology

4.1 Introduction to mushroom biotechnology

For thousands of years, humans have employed microorganisms unconsciously. They have used them through baking and brewing practices since the earlier times without fully understand the concept behind. Knowledge on microorganisms did not expand much before the 19th century when the connection was made between them and the fermentation process.
The term fermentation refers not only to alcohol production by yeast but on all microbial biochemical transformations with a significant commercial use. Baking was one of the very first kind of microorganism exploitation by using *Saccharomyces cerevisiae* to ferment dough made out of wheat. The fermented sugars releases carbon dioxide causing the dough to rise, ethanol is produced and kills the yeast before being expelling during the baking process. [29]

The deeper understanding of fungi revolutionized the business of biotechnology and had a huge impact on several industries. At the present time, growth parameters to produce microorganisms are more understood than ever and new applications are constantly found. Currently, a major industrial purpose to the industrial microbial processes is the production of enzymes.
Enzymes are green alternatives to industrial catalysts. They are employed in many areas such as food and beverages for juice liquefaction (xylanase, pectinase), detergent formulations (proteases, lipases) and biomass hydrolysis for biofuels (cellulases, xylanases). Proteases were the highest selling enzyme until 2012 but the trend shifted to carbohydrases in 2013. The shift occurred due to the increase in sales for research toward the second-generation biofuels. [40]

4.2 Industrial enzymes

In Nature, microorganisms are breaking down cellulosic material to use cellulose as energy and carbon source. Numerous fungi, bacteria and actinomycetes are participating in the recycle of the biomass. Due to the recalcitrance of cellulosic material, the breakdown rate in nature is slow. Microorganisms are able to degrade cellulose and hemicelluloses thanks to their enzymes system of production which release them into the environment. [29]

Enzymes hydrolyzing β-1,4 linkages in cellulose chains are called cellulases. They generally possess noncatalytic carbohydrate-binding modules (CBMs) and/or another module located at the C- or N-terminus of the catalytic module. There are three main types of cellulolytic enzymes: endoglucanases, exoglucanases and glucosidases. Crystalline cellulose can only be degraded when the cellulase system of the microorganism is complete (ie when the three types are produced). Generally, most bacteria possess an incomplete system but some fungi produce the three types of cellulolytic enzymes. Most aerobic cellulolytic microorganisms secrete a set of individual cellulases where CBM is joined to the catalytic module by a flexible linker peptide. In comparison, most anaerobic microorganisms produce cellulosomes, large multienzyme complexes bound to the surface of the cell [29][33]. **Figure 8** represents the degradation of cellulose and xylan by the different enzymes systems.

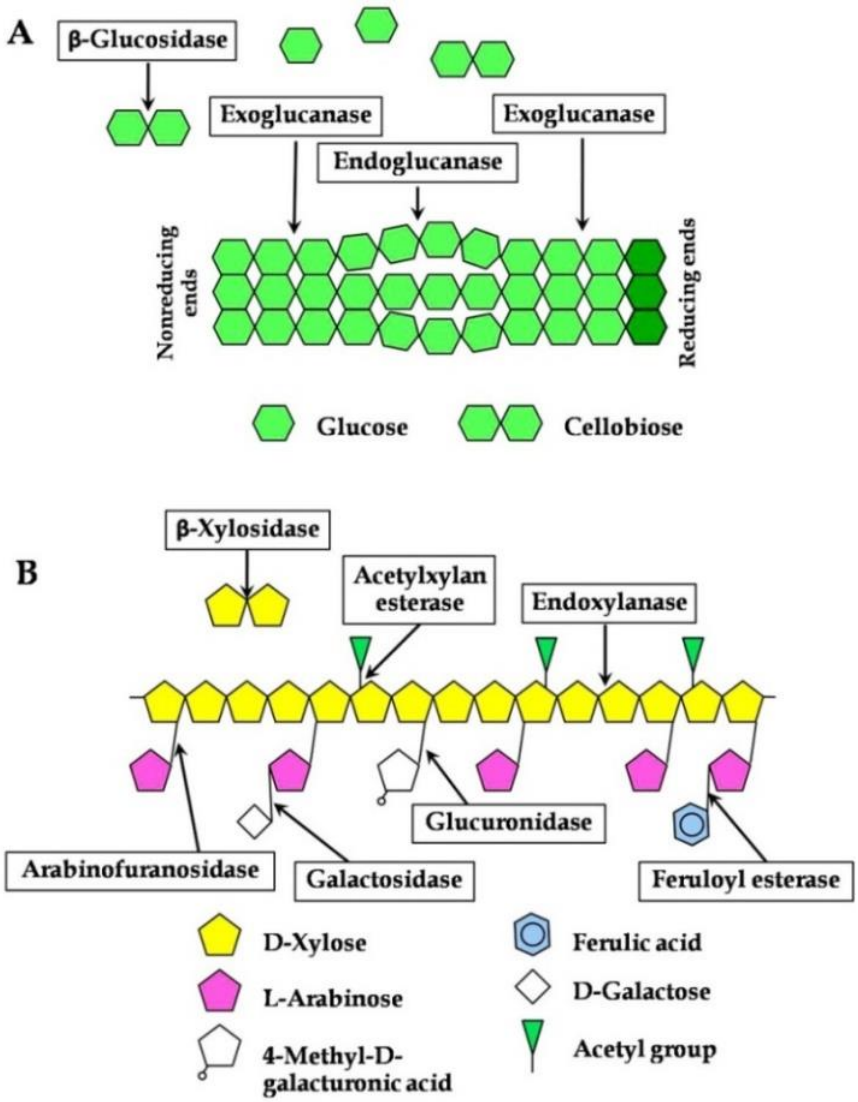

***Figure 8**: Enzyme system involve in the degradation of cellulose (A) and xylan (B). [46]*

4.2.1 β-glucosidases (BGs)

Enzymes such as β-glucosidases (beta-D-glucoside glucohydrolases) are well characterized and widely studied enzymes. They are biologically important by playing a role in various biotechnological processes such as the production of biofuels, ethanol and hydrolysis of isoflavone glucosides. BGs are widely found in nature (plants, fungi, bacteria and animals) and their importance is considerable in many biological processes. Depending on the biological system, and the location of the enzyme, the physiological purpose associated will vary. Their role in cellulolytic microorganisms is mainly cellulose hydrolysis and cellulases induction by converting cellobiose into sophorose (which is an inducer in some microorganisms) by transglycosylation. BGs are involved in cell wall development in plants through the synthesis of beta-glucan, fruit ripening and also in defense mechanisms. Their role in humans and mammals organisms is to hydrolyse glucosyl ceramides. [38][51]
BGs catalyze the hydrolysis of the short chains of oligosaccharides, disaccharides, alkyl and aryl-β-glucosides. Cellobiases are crucial for their hydrolytic and synthetic activities for recent biotechnological applications. Efficient and complete saccharification of cellulose is primordial to make the biofuel production economically viable. Therefore, cellobiose which is an inhibitor of endo-glucanase and exo-glucanase has to be removed through the action of cellobiases. [39]

Activities of BGs are measured through the use artificial substrate such as PNPG (para nitrophenyl beta-D-glucopyranoside) preferentially to cellobiose. This is because the binding patterns toward the two substrates are different as cellobiose needs a conformational change contrary to PNPG. Therefore BGs show high catalytic activities and high Km with PNPG and not with cellobiose. Endoglucanases, exoglucanases and β-glucosidases act synergistically in order to efficiently hydrolyze cellulose. [35]

4.2.2 Xylanases

Xylanases are enzymes able to hydrolyze the linear polysaccharide xylan (the main component of hemicelluloses) into monomeric sugars. They belong to the glycosyl hydrolase family and include endo-1,4-β-xylanase, β-xylosidase, acetylxylan esterase and α-arabinofuranosidase. The first is particularly important as it catalyzes the hydrolysis of β-1,4 linkage in xylan, breaking the backbone of the chain for further hydrolysis. The official name is endo-1,4-β-xylanase but

they are found under various names including xylanase, β-xylanase or endoxylanase. Species like *Penicillium*, *Aspergillus* or *Trichoderma* secrete cellulolytic enzymes in parallel of xylanase. [38]

Xylanases produced from microorganisms are advantageous in industrial applications. They provide a lot of advantages like utilization of cheap carbon sources, closed systems for production, rapid multiplication and low-cost production. Many potential applications will be discussed further. The constitutive production of xylanolytic enzymes has already been reported but most of them are inducible in nature. Xylan will be used as a substrate to grow the microorganism and as inducer to produce xylanases. Cellulose, methyl-D-xyloside and synthetic alkyl are other inducers of xylanases. Monomeric sugars like glucose and xylose both act as catabolic repressors. Xylanases can be produced using submerged fermentation (SmF) and solid-state fermentation (SSF). [38]

4.2.3 Source of (hemi)cellulolytic enzymes

Although many living beings are carbohydrate degraders, not all are able to degrade cellulose and hemicellulose. For most of them (hemi)cellulose digestion is only possible through their microflora. Microorganisms are almost the only living beings capable of producing (hemi)cellulases in nature. Cellulose degraders can be found in both aerobic and anaerobic bacteria usually found in soil, water and on plant materials but the most studied cellulose producers are fungi. [38]

Fungi ability to secrete external cellular complex and higher enzyme production make them more suited than bacteria for an industrial purpose. The most commonly studied fungi include *Trichoderma*, *Aspergillus* and species from *Penicillium*. All three are known to produce cellulases as well as xylanases. These filamentous fungi are already used in enzyme industries. The controlled production in closed system, the low cost of production, and the possibility to use cheap material as carbon sources is why microbial cellulases and xylanases are preferred for industrial applications. At the present time, *Trichoderma reseei* is reported to be the best cellulase producer. However many other fungi are reported as potential enzyme producers. The majority of (hemi)cellulolytic organisms cannot be cultured thus the isolation and characterization of microorganisms from nature is very limited. [38]

4.3 The solid-state fermentation

Enzyme production by microorganisms may be achieved either by submerged fermentation or by solid-state fermentation. SmF involves the growth of microorganism and substrate in flowing water compare to SSF which involves the growth of the microorganism on dry substrate with almost no free water. Both methods have advantages and disadvantages.

At the moment, the industry generally chooses to produce enzymes in SmF because of the ease to control the whole process parameters. Its use began in the 1940s with the increasing need of large-scale production of antibiotics. Aeration, agitation, temperature and pH are some of the well-controlled parameters. Reactor technology permits to extensively control those parameters which are essential for the production of enzymes. Therefore scaling-up to bigger reactors is not so difficult since they are commonly used in the industry. Moreover, the dissolved oxygen needed for the growth of microorganisms can homogeneously be dispersed. Yields of the product of interests are directly linked to these important parameters (**Figure 9**). [42]

Unlike SmF, SSF mimics much more the natural habitat of the microorganism. Fungi growing on dry substrate develop their mycelial form, producing substrate penetrating and aerial hyphae.

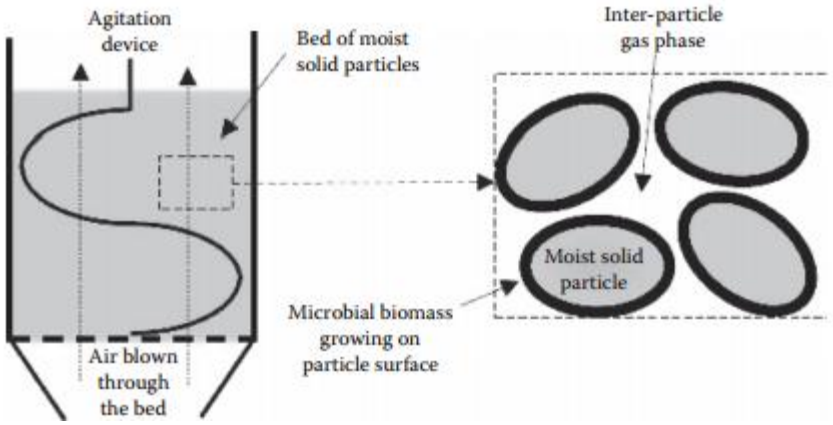

***Figure 9**: Typical physical process of oxygen transfer in a SSF bioreactor. [43]*

Even though SmF is easier to implement in an industrial scale, many scientific papers reports higher yields for the same strain growing in SSF [44]. Considering historical aspects, SSF processes have been used in Asian countries for a long time, particularly in the food industry. For example, *Trichoderma* strains are used in the saccharification of rice for the production of sake, an alcoholic beverage. Some thousand-year-old SSF processes are used to produce enzymes and metabolites for fermented food. SSF survived through the ages via knowledge transmitted from father to son as handmade processes. Western countries in comparison have

almost abandoned it due to industrial standardization, rationalization and the need of scaling. [42][41]

Fermentation on solid substrate has plenty of benefits. It simulates a natural environment resulting in an improvement of the growth of the microorganism. Some metabolites and enzymes such as mycomycin and conidiospore cannot be secreted by liquid fermentation. The fermentation medium is simpler and does not need as much energy as liquid fermenters. The water consumption being far lesser, the process is therefore greener and has a lower impact on the environment. SSF fermenters are simpler and less expensive than liquid bioreactors. Basically solid-state fermenters only need space with temperature control in order to be implemented. Downstream processing (mainly purification) is easier to manage since the product of interest is recovered in higher concentration. [37][44]

However, some downsides are worth mentioning. Heat generated by metabolic activity of the microorganism can hardly be removed. Aside from controlling the airflow and the chamber temperature, not much can be done and the bioreactor can easily overheat. [37]

4.4 Solid-state fermentation parameters

Many components may affect the growth of the microorganism. The SSF system is a mix of gas, liquid and solid phases. Water activity, temperature, aeration, pH and the choice of a solid substrate are the most important parameters for enzyme production.

- Water activity and moisture content

Water activity of the substrate is a critical parameter for microorganisms. Enough moisture is necessary to support its growth and its metabolism.

Water activity (a_w) is defined as:

$$a_w = \frac{p}{p_w}$$

Where p is the saturation vapor pressure of humid substrate and pw is the saturation vapor of water at the same temperature.

The optimal a_w differs from the growing microorganism and the species. Bacteria generally grow with an a_w value ranging from 0.90 to 0.99 and yeasts tolerate some lower values (up to 0.8). Fungi need even less water activity as they can grow at an a_w between 0.6 and 0.7, making

them well suited for a SSF where a_w is pretty low. Another benefit of a low water activity is the prevention of a bacterial infection in the bioreactor. [44]
The environment with a high a_w prevents the diffusion of carbon dioxide and oxygen, reduce the porosity of the substrate and increase the likelihood of bacterial development, thus resulting in difficulties for the fungi to grow. Water activity plays a significant role in enzyme production. Pectinase and xylanase activities were found to be much higher when moisture was under 65%. The low activity at high substrate moisture could be attributed to a decrease of porosity, the gummy texture resulting, or even a lower oxygen transfer. Furthermore, a lower moisture level impacts the diffusion of nutrients in the substrate. [44]

- Effect of temperature and Aeration

One of the most important variables in the microbial growth is the temperature. Spores forming, product formation, germination and growth of spores are parameters affected by high temperature. Heat transfer is a difficult issue to face for the scale-up of SSF processes since enzyme denaturation can occur due to aerobic conditions which produce metabolic heat. This heat is difficult to control since the SSF occurs without free water unlike in SmF. Each microorganism has its own kinetic of growth and product formation at a specific temperature. The optimal temperature can hardly stay so especially during the logarithmic growth phase. Temperature issues may be limited with the implementation of forced aeration, resulting in a better control of heat transfer for a large-scale SSF. Many studies have focus on optimal temperature for the production of (hemi)cellulolytic enzymes and have come to conclusion that optimal growth temperature for fungi generally varies between 30 and 40°C. [44]

- Effect of pH

The overall efficiency of the fermentation process is highly influenced by the pH in the culture media. This is because the metabolic activities that are taking place during the fermentation process change in response to the pH of the medium. The secretion and uptake of organic acids cause the pH to vary during fermentation. Even though pH is an important parameter, its monitoring and control in SSF is not simple. Most of studies of enzymes production only report the influence of the initial pH of the medium. The pH range for the enzyme production is about 4 to 5 for optimal yields. [45]

- The solid substrate

The two main factors being considered for selecting the appropriated substrates for SSF are cost and availability. A major advantage of fermentation on solid substrate is the possibility to use agro-industrial wastes. These byproducts of the industry are generated in large amount with a low commercial value. Among many substrates, coffee pulp, wheat straw, rice straw and cereal bran can be used as solid substrates. The goal of the solid substrate is to provide energy as a carbon source. Their properties are determined by the basic structures of the substrates (starch, pectin, fiber, hemicellulose, cellulose and lignin). The growth medium must provide the limiting macro and micronutrients such as sulfur, nitrogen, potassium, magnesium, zinc, copper, iron, iodine, cobalt, sulfur and phosphorus to sustain the growth of the microorganism. [37]

Moreover, physical properties such as surface area, crystallinity and accessible area of the solid substrate play a significant role on the SSF process. Among many lignocellulosic materials, wheat bran and wheat straw are some effective substrate for the enzyme production. Undoubtedly, the SFF technology can by a wide range of agro-industrial residues to produce (hemi)cellulolytic enzymes for the bioenergy sector. [45]

4.5 Biotechnological production of enzymes using agro-industrial residues, market and industrial applications of enzymes

A sustainable alternative of fossil-fuel-based products can be achieved through biorefining. Lignocellulosic biorefineries are able to produce a wide spectrum of marketable bio-based products with the help of microorganism's enzymatic activity. Plant polysaccharides coming from nonfood biomass are processed into monosaccharides to become the first platform chemical for plastics and fuels. A sustainable form of energy worth mentioning is the production of biohydrogen from organic wastes by photo- and dark fermentation. This is only possible because of the efficient fermentation by microorganisms of low-cost and renewable substrate. Actually, about 10.2% (50.3 EJ/year) of the annual world energy supply is provided by a wide variety of biomass sources. Each year across the world, some 140 billion tons of biomass are generated from agriculture sectors [32][31]. According to the International Energy Agency (IEA), 50% of the world energy needs could come from biological supplies. Industrial enzyme applications are constantly increasing. They are used in a large variety of industries such as food, textiles, detergents and the pulp and paper industry.

More specifically, industries are also looking for xylanases in baking, to process biomass for biofuels, for de-inking and for animal feed. Enzymatic bleaching is a good alternative to chemical bleaching which generate highly persistent chlorinated organic compounds, causing great arms to the environment by causing toxic and mutagenic effects. Endo-1,4-β-xylanase are particularly interesting for their usage under extreme temperature and pH conditions. [33]

Multiple factors are stimulating the fast growth of the enzyme market. The need of new renewable technologies, particularly for the conversion of lignocellulosic biomass into bio-ethanol is one of the principles. Among the important parameters to commercialize lignocellulose-based enzymes is the need to reduce the costs of enzymes productions and increase their catalytic efficiency of the process to enhance the economic feasibility. [33]
Thus, using agro-residues as substrates for enzyme production is a profitable alternative. These unwanted wastes are given a second life and the amount of investment made per batch of enzymes produce is very limited. [40]

II. Objectives

The main objective of the study is the evaluation of by-product of the hemp industry for the production of fungal enzymes by solid-state fermentation. Hemp shives and inflorescences are investigated as potential lignocellulosic carbon source for solid-state fermentation. Substrates will be fully characterized in order to study the enzyme yields according to the chemical composition.

Steam explosion will be carried out to achieve two goals in one step. The first is the extraction of the secondary metabolites of hemp inflorescences. The second is to apply a pretreatment on the biomass in order to overcome the physical (low porosity and contact surface) and chemical barriers (cellulose crystallinity, lignin) of the lignocellulosic matrix. These barriers must be overthrown to increase the accessibility of cellulose and enhance both hydrolysis and fermentation. The steam explosion pretreatment will be carried out at 10 and 20 bar (~200°C and 215°C, a few minute residence time). Solid and liquid fractions will be chemically characterized and the effect of the solid fraction on enzyme production investigated.

Two kinds of fungal strains (*Sarocladium kiliense* CTGxxyl and *Penicillium canescens CWBI-F58*) are selected as potential enzyme producers. Solid-state fermentation is conducted during one and two weeks on both hemp inflorescences and hemp shives. Xylanase and β-glucosidase are investigated as potential enzyme products. These green-based enzymes would meet the need for sustainable solutions in industrial bioprocesses.

III. Materials and methods

5 Raw materials

Different parts of industrial hemp *(Cannabis sativa L)* plant were used throughout this study. Hemp cultivars have to be approved by the European Union for cultivation. Authorized cultivars are not allowed to produce more than 0.2% Δ^9-THC in the whole plant and have to reach a ratio of $\frac{CBD}{THC} > 1$ according to the United Nations Office on Drugs and Crime (UNODC). Growing hemp in Belgian requires special authorization. Raw material was provided by Belchanvre, a Belgium cooperative harvesting and processing hemp. Hemp inflorescences from the strain *Finola* were harvested on the 19th august 2016 and were then stored in a dry place. Woody core (shives) was separated from the fiber in a decorticator facility in Marloie (province of Luxembourg). Shives were roughly chopped into hemp chips. Fresh inflorescences (flowers and seeds) were specially harvested for study purpose. Materials was stored in a dry place for 6 months before its use. Wheat straw was harvested during the year 2016 and stored in a dry place as well.

6 Steam explosion pretreatment

Hemps hurds and inflorescences were both pretreated by Steam explosion in prototype pilot scale equipment with a 50L reactor volume (Jacquet et al., 2011[23]). The apparatus is composed of different parts: a steam generator (29.4 kW) with an operating pressure at 6.0MPa, a 50L reactor with maximum pressure capacity of 5.1 MPa an explosion tank where the "explosion product" is collected. The instant release of pressure needed for explosion effect is triggered by a quick-opening ball valve situated between the reactor and the explosion tanker.

500g of hemp hurds were pretreated in triplicates by Steam explosion at 10 and 20 bar (respectively 200 and 215°C). 250g of flowers and seeds were exploded in single sample at 10 and 20 bar (200 and 215°C). Repetition was not affordable due to the low amount of raw material. The biomass was collected from the tanker with water then dried in an oven at 50°C and stored in a dry place. 1L of distilled water was added to the reactor to impregnate the biomass. Residence time of the substrate in the reactor slightly varied between each batch

(from 45s to 2'45s). The explosion was triggered at the time the desired pressure was reached in the reactor. Pretreated hemp shives in the reactor were collected with water then separated by centrifugation with a Centrifugator Rousselet Robatel RC 40 VX at 15000 rpm. Steam exploded hemp hurds were washed by 5L of water before being dried at 50°C for 24h and stored in a dry place at room temperature.

7 Fractions characterization of the biomass

Chemical analyses were performed in triplicates. Steam exploded hemp shives samples were characterized by means of the three steam explosion replicates. Inflorescences and wheat straw were characterized with single samples in triplicates.

Dry matter quantification was based on the National Renewable Energy Laboratory (NREL) method [58]. 0,5 g of sample were heated at 105°C for 24h in an oven (Memmert SNB 100) then weighted. Moisture was calculated relative to the dry mass of the original material.
Ash content was determined gravimetrically as the solid fraction remaining after a 4h combustion at 575°C in a muffle furnace (Nabertherm controller b180). [53]

Protein content was estimated according to the Kjeldhal method by measuring the nitrogen content. Instrumentation used consisted in a TECATOR 1015 for sample mineralization and a Kjeltec 2300 (Foss) for titration. Results were multiplied by a conversion factor of 6.25 used as suggested for animal feed and other materials. [54]

Extractives were quantified by using a Soxhlet apparatus as described in the NREL method [55]. A 1g sample of raw material was successively extracted by 70 mL of water during 6 hours and 70 mL of ethanol during 12h. Soluble compounds (organic acids, secondary metabolites, etc…) from both extractions were quantified gravimetrically. **Fat content** was extracted from a 2.5 g sample by 70 mL of hexane in a Sohxlet apparatus for 12h. Fat content was determined gravimetrically after removal of the solvent. Results were expressed as a percentage of the raw material, on a dry matter basis. [77]

Lignin content was determined according to the Klason method (Sluiter et al., 2012). 3 mL of H_2SO_4 (72%) were added to 300 mg samples of raw material. After 1 hour of reaction time, 84 mL of water were added to a Schott tube then placed in an autoclave for 1 hour at 121°C.

After filtration, the remaining solid was dried for 6 hours at 105°C before being weighted. "Insoluble lignin" was calculated relative to the dry mass of the original material. The protocol was followed as described in the NREL method [56]. "Soluble lignin" content was measured by spectrophotometry using a UV-1800 Shimadzu spectrophotometer at λ 205 nm according to the method from [Korpinen et al, 2014]. Total lignin content was assumed as equal to the sum of soluble and insoluble lignin.

Total sugar content (free monosaccharides, cellulosic fraction and non-cellulosic polysaccharides) from the different samples was determined after free water soluble monosaccharides (glucose, xylose, mannose, arabinose, galactose, rhamnose) were extracted at 50°C for 30 min. Chemical hydrolysis was performed with H_2SO_4 1M at 100°C during 3h in order to convert non-cellulosic polysaccharides into monosaccharides. Cellulose hydrolysis was performed in 2 sequential parts. The first hydrolysis was performed using H_2SO_4 72% at 30°C for 1 hour. The second hydrolysis was carried out by diluting the acid to reach 1M, reacting for 6 hours at 100°C.

1-Methylimidazole and acetic anhydride were used to derivatized monosaccharides into alditol acetates prior to the gas chromatography analysis [57]. Agilent (7890B series) gas chromatograph was used to carry out the analysis. Components were separated using a capillary column (HP1-methylsiloxane 30m x 0.32 mm ID, 0.25 μm thickness). A flow rate of 1.6 mL min-1 of Helium as carrier gas was used and injection chamber temperature was set at 290°C. Temperature plate was set at 120°C for 1 min followed by a linear increase to 290°C in 35 min. A flame ionization detector was used at 320°C. Data analyzes were performed using OpenLab Chemstation software. Internal standards (glucose, arabinose, mannose, galactose, xylose, rhamnose and 2-deoxyglucose) were bought at Sigma-Aldrich. In order to obtain polymeric sugars, an anhydro correction of 0.90 for hexoses and 0.88 for pentoses were applied to the concentrations of monomeric sugars.

Free monosugars and degradation products such as 5-hydroxymethylfurfural, 2-furfural (2-F), formic acid, acetic acid, lactic acid, levulinic acid) were determined at the surface of the biomass as well as in the washing effluents from the steam explosion. 500mg of solid fraction were stirred in 10 mL water for 30 min at 50°C. Analyses were carried out on the liquid extract. Derivatization technique applied for total sugar content determination was applied to the free monosaccharides liquid extract prior to the gas chromatography analysis.

Degradation products (acetic acid, formic acid, lactic acid, levulinic acid, 5-HMF and 2-F) were determined in the solid and liquid fractions of steam exploded samples with a HPLC (Waters 2695) device. Before the injection, samples were filtrated with a filter at 0.45µm. The method designed for steam exploded samples was using an Aminex HPX-87H (300 x 7.8 mm) (Bio-Rad) column. H_2SO_4 5 mM was used as eluent at a flow rate of 0.6 mL/min at 45°C during 60 min per sample. Standards for calibration curves were purchased from VWR Chemical.

Elemental analyses (organic carbon, P_2O_5) were performed by the Bureau of Environmental study and Analyses (BEAGx) and were given as percentages of Corg and P_2O_5 present in the dry samples.

8 Solid-state fermentation

8.1 Microorganisms and culture media

Sarocladium kiliense CTGxxyl and *Penicillium canescens CWBI-F58* were used for the production of enzymes. The strain *Sarocladium kiliense* was isolated from termites fed on xylan while the strain of *Penicillium canescens* came from the Institute of Plant Biomestry (Academy of Sciences, Tbilisi) in Georgia. Both strains were grown on potato dextrose agar (PDA) medium for 4 days in controlled environment at 30°C. PDA concentration was of 39g/L in deionized water adjusted to pH 7. The solid-state fermentation medium was placed in a 250 mL Erlenmeyer flask containing 2.5g of substrate. Considering the substrate morphology, the amount of medium to impregnate the hemp inflorescences (5mL) had to be of 1/3 of hemp shives (15 mL).

The **fermentation medium** on **hemp hurds** was adapted from (Bakri et al., 2003[59]) consisted of: 10 g/L Na_2HPO_4 + 0,5 g/L KCL + 0,15 g/L $MgSO_4.7H_2O$. Additional nutrients were added to induce the enzyme synthesis. 0.4g/L of yeast extract and peptone were added as nitrogen sources. Additional trace metals from (Mandels et al, 1969) [60] middle were added: 5mg/L $FeSO_4.7H_2O$, 20 mg/L $CoCl_2$, 1.6 mg $MnSO_4$ and 1.5 mg/L of $ZnSO_4$.

The fermentation medium on **inflorescences** was similar but concentrated 3 times: 30 g/L Na_2HPO_4 + 1,5 g/L KCL + 0,45 g/L $MgSO_4.7H_2O$, 1.2g/L of yeast extract, 1.2g of peptone. Trace added metals: 15mg/L $FeSO_4.7H_2O$, 60 mg/L $CoCl_2$, 4.8 mg $MnSO_4$ and 4.5 mg/L of $ZnSO_4$. The pH was adjusted to 5 before 2 hours sterilization in an autoclave at 121°C.

8.2 Substrates and inoculum

Flasks were inoculated aseptically with a **spore suspension** (10^6 spores/mL) and then incubated at 30°C for 7 and 14 days.

Solid-state fermentation was performed on hemp shives and hemp inflorescences, on untreated material and material pretreated by steam explosion at 10 and 20 bar (200°C and 215°C). Spores were harvested by washing the plate with 9 mL of sterile water. Bürker counting chamber was used to determine the number of cells per milliliter of liquid medium for inoculation. Media were inoculated with a spore suspension to a final concentration of 1×10^6 cell/mL and 3×10^6 cell/mL in order to keep the parity with the nutrients concentration. Fermentation conditions were carried out in duplicate. Flasks were incubated in a stove at 30°C for 7, 14 or 21 days.

Enzyme extraction was carried out with 20 mL of citrate buffer with 2mL/L of tween 80. Tween 80 is used to unhook the enzyme from the substrate. Erlenmeyer flask was agitated for 1h at room temperature at 80 rpm before filtration on a nylon bag to separate the raw biomass from the liquid extract. Liquid extract was then centrifuged at 5000 rpm for 10 min in a Fisher bioblock scientific 2-16P apparatus prior to freezing for conservation.

Enzyme assay was based on liquid extracted from the solid-state fermentation. Enzyme activities were determined by colorimetric methods. All liquid extracts were analyzed in duplicates. **Cellobiase activity** was estimated from 1mL of sample mixed with 1 mL of PNPG (2.5.10-3 M) in phosphate buffer at pH 5.85 in a test tube. The whole was then incubated at 40°C for a 15 minutes reaction. Reaction was stopped by adding 1 mL of Na_2CO_3 (1M), diluted with 20 mL of water then 1mL of supernatant was collected and centrifuged at 13400 rpm for 5 min. Optical density was read at 550 nm. Calibration curve was set with paranitrophenol (10^{-5} to 10^{-4} M). Enzymatic activity was estimated after withdraw of the substrate blanco as well as enzymatic blanco.

To determine the **endoxylanase activity**, 0.25 mL of enzymatic solution was added to react with 1.75 mL of xylan solution (10g/L). Xylan substrate was prepared with phosphate buffer at pH 6.5. After 5 minutes of incubation time at 40°C, reaction was stopped by 3,5-dinitrosalicylic acid (DNS). DNS is generally used as a method to calculate the enzyme activity by measuring the reducing carbohydrates released by the reaction. Incubation at 100°C is needed to estimate

the xylanase activity. After diluting with 10 mL of water, 1 mL was withdrawn in an Eppendorf then centrifuged at 13400 rpm in a Minispin Eppendorf Microcentrifuges.

Statistical analyses were performed on enzymatic yields with Minitab 16 software. One-way ANOVA was performed to differentiate de means. Two-way ANOVA model was performed taking into account the steam explosion pretreatment efficiency on the substrate in regards to the enzymatic production.

IV. Results and discussion

The first part of the results concerns the characterization of hemp shives and hemp inflorescences that are used as substrates for solid-state fermentation, the steam explosion pretreatment and its effect on the chemical and elemental composition of the substrate. Afterward, fermentable free sugars and degradation products (5-HMF, 2-F and organic acids) in the liquid and at the surface of the solid fraction are discussed.

The second part concerns the enzyme productions during the solid-state fermentation. Yields in enzymes production are discussed in regards to the substrate, the severity of the treatment and the microorganisms that are used. Enzymes productions are related to the composition of the substrate being used as support and carbon source for the microorganism. This early conclusion on the substrates is used for further modifications to improve the solid-state fermentation in order to increase the enzymatic yields. A last discussion determines whether or not the modified parameters are successfully changed and then a general conclusion is drawn.

9 Biomass characterizations

Table 1 shows the chemical composition of hemp shives, hemp inflorescences and wheat straw (control biomass) being used as substrates for SSF. The chemical composition of the biomass is extremely important in regards to their use in this kind of process. The substrates is mainly used by microorganisms as carbon and nitrogen sources for growth and impacts the enzymatic production. Therefore, the substrate composition is supposed to play a significant role as a source of nutrients for microorganisms. Polysaccharides and proteins are available only for the microorganism to use by converting them into monosaccharides and amino acids with its enzymes production. Another key factor is the lignin content which remains one of the main obstacles to the access of the complex lignocellulosic matrix. The respective dry matters were of 93.2 ± 0.1 % for the hemp shives, 93.1 ± 0.4 % for the hemp inflorescences and of 94.0 ± 0.1 %) for wheat straw.

***Table 1**: Chemical composition of hemp shives, hemp inflorescences and wheat straw.*

Components[1]	Shives wt% dry basis	Inflorescences wt% dry basis	Wheat straw wt% dry basis
Polysaccharide			
Rhamnan	0.1 ± 0.1	0.5 ± 0.1	0.3 ± 0.0
Arabinan	n.d.	0.5 ± 0.3	4.3 ± 0.4
Xylan	9.1 ± 1.4	5.7 ± 0.7	19.3 ± 0.6
Mannan	1.0 ± 0.0	0.3 ± 0.7	1.1 ± 0.3
Glucan	32.2 ± 4.7	11.4 ± 1.5	25.9 ± 1.7
Galactan	0.5 ± 0.1	0.8 ± 0.2	1.4 ± 0.0
Extractives			
Water extractives	6.5±0.7	18.0 ± 0.3	19.5 ± 0.9
Ethanol extractives	4.0 ± 1.3	18.0 ± 2.3	3.0 ± 0.8
Lignin			
Insoluble lignin	22.9 ± 2.5	22.2 ± 2.0	11.6 ± 0.3
Soluble lignin	2.0 ± 0.3	5.2 ± 0.5	3.4 ± 0.6
Proteins	2.0 ± 0.0	21.0 ± 0.3	4.9 ± 0.1
Ash	11.0 ± 0.1	2.9 ± 0.1	7.6 ± 0.2

[1] Analyses were performed in triplicates

Chemical compositions are reported on dry basis weight. Hemp shives and wheat straw both have glucan as main constituent (32.2 ± 4.7 % and 26.0 ± 1.7 %) but the second has a high content in xylan as well, being therefore a good candidate for xylanase production. Hemicelluloses proportion in shives (10.7 ± 1.6 %) may seem undervalued considering some concentrations found in the literature which were around 25-35%. However, some studies reported very low concentrations (5.5%) which support the present data [65]. Lignin and protein content are very similar to percentages found in by [63][64]. However, collected ashes are much more important than in those reported (1-3 %) by these studies but it was already noticed that hemp cultivars composition varies from one to another.

Inflorescences contain a low amount of polysaccharides (19.2 ± 2.5 %) which would make it less suitable for enzyme induction. The high content of proteins coming from the seeds is a precious nitrogen source for the growth of the microorganism. Lignin is one of the main constituent in both inflorescences and shives (respectively 27.7 ± 2.5 % and 24.9 ± 2.8 %). A pretreatment method was carried out in order to overcome the complexity of the

lignocellulosic material and enhance the enzymatic hydrolysis and fermentation. Classical hemp inflorescences are hardly comparable to this one because usually the characterization is done on flowers or seeds separately.

9.1 Steam explosion pretreatment

In the present work, the steam explosion pretreatment was applied on the biomass aiming two separate goals. On one hand, to valorize a by-product of the industrial hemp industry (shives) which has not found a real market yet. On the other hand, steam explosion was investigated as a possible extraction method in order to collect the high added value molecules (cannabinoïds) from the hemp flowers. However extraction results were not investigated in the present work. The remaining treated biomass was used as SSF substrate for the production of endoxylanase and cellobiase.

The steam explosion parameters (time for the boiler to reach the required pressure and temperature) slightly varied during the biomass pretreatment. **Table 2** depicts the STEX parameters for each batch of hemp shives as well as the resulting severity factor. Exact time to reach the target pressure (10 and 20 bar) before the explosion for inflorescences could not be estimated due to a loss of data caused by a power failure.

***Table 2**: Steam explosion pretreatment conditions on hemp shives.*

Batch n°	Pressure (bar)	Time (min)	Temperature (°C)	Severity factor (Ro)
1	20	7.5	215	3.2
2	20	2.7	215	2.8
3	20	1.3	215	2.5
4	20	1.3	214	2.4
5	10	0.8	202	2.2
6	10	1.0	186	2.3
7	10	0.6	211	2.1

Depending on the time intervals between two explosions and different washing conditions of the reactor and tanker, initial temperature of the boiler may vary, resulting in a different residence time of the biomass in the reactor. **Batch 1** had the most severe treatment. Even though temperature and pressure parameters are linked, those two do not match exactly.

Residence time is a preponderant parameter compared to temperature. For example, by comparing **batch 6** with **batchs 5** and **7**, those two have reached a higher temperature for a shorter amount of time and the resulting severity factor is lower. **Batch 1** was a preliminary test to heat up the SE apparatus. Since the collected raw material was still heavily loaded with liquid water compare to the 6 other batches which had almost dry material coming out, the decision was made not to use it for further experiments.

The STEX pretreatment is a combination of physico-chemical effects of temperature, steam and mechanical disruption. The resulting shearing of the biomass has an impact on its aspect with a completely different outcome for shives and flowers. **Figure 10** gives a good insight of the different morphology of the substrate that was used during the experiment. Shives treated at 10 bar (Sh10) were slightly exploded but remained as raw chips from the untreated material. However, shives exploded at 20 bar (Sh20) were much more airy and voluminous (visible on fig.1), leading to an increased contact surface of the raw material with the microorganism. The washing water of the shives was a deeply brown liquid with a heavy load of degradation products. Flowers and seeds came out of the tanker as a black compact cluster which had to be milled for ease of utilization. These morphological differences had consequences on the ability of the substrate to soak water.

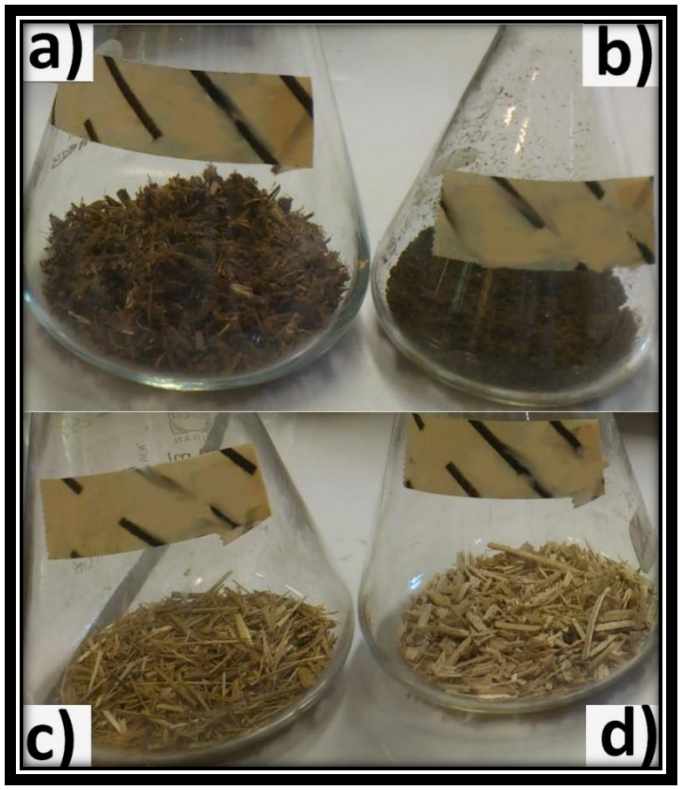

***Figure 10**: 2.5g of SSF substrate including pretreated hemp shives at 20 bar (a), milled hemp inflorescences (b), wheat straw (c) and untreated hemp shives (d).*

The time of pretreatment, the temperature and the granulometry of the substrate directly interfered with the biomass recovery. Among the pretreated substrates, Sh10 and Sh20 lost an average of 46 % and 26 % of their mass respectively, while In10 and In20 lost about 21% and 90%. Most of the biomass stayed stuck in the tanker and the conduits. Some of the material stays in the reactor and cannot be removed before 1 or 2 explosions with water. Sh10 poor recovery is probably due to a lack of pressure to expel the biomass from the reactor to the tanker. The amount (250g) and the light weight of the inflorescences might not be enough to get a decent recovery at 20 bar. Most of the biomass was probably stuck to the wall of the tanker and reactor.

The respective dry matter percentages of the biomass are the following: Sh10 (94.7 ± 0.2 %), Sh20 (94.7 ± 0.1 %), In10 (97.2 ± 0.4 %) and In20 (97.1 ± 0.6 %). **Table 3** provides the percentages in polysaccharides, lignin, proteins, ash, fat and extractibles found in the biomass. Wheat straw composition has been added to the table to discuss further results in the fermentation part.

***Table 3**: Chemical composition of shives and inflorescences pretreated by steam explosion.*

Components[1]	**Shives* (10 bar)** wt% dry basis	**Shives* (20 bar)** wt% dry basis	**Inflorescences** (10 bar)** wt% dry basis	**Inflorescences** (20 bar)** wt% dry basis
Polysaccharides				
Rhamnan	n.d	n.d	n.d	n.d
Arabinan	n.d.	n.d	0.1 ± 0.1	n.d
Xylan	9.7 ± 0.1	8.0 + 2.6	3.7 ± 0.6	5.4 ± 0.4
Mannan	0.8 ± 0.0	1.0 ± 0.2	0.6 ± 0.1	0.5 ± 0.2
Glucan	31.7 ± 1.0	44.7 ± 7.0	13.4 ± 2.2	15.9 ± 2.7
Galactan	0.2 ± 0.1	0.1 ± 0.1	0.6 ± 0.1	0.4 ± 0.3
Extractives				
Water extractives	4.9 ± 0.1	7.6 ± 6.2	5.5 ± 0.4	5.8 ± 0.3
Ethanol extractives	7.4 ± 1.1	10.2 ± 2.7	27.3 ± 1.9	27.3 ± 1.9
Lignin				
Insoluble lignin	23.3 ± 2.1	21.8 ± 1.5	17.8 ± 0.8	24.8 ± 3.3
Soluble lignin	2.6 ± 0.4	2.4 ± 0.5	3.4 ± 0.4	6.0 ± 0.3
Proteins	2.0 ± 0.0	2.0 ± 0.0	24.3 ± 0.5	20.7 ± 0.1
Ash	10.0 ± 0.2	9.0 ± 0.2	1.6 ± 0.2	1.4 ± 0.2

[1] samples were analyzed in triplicates

*samples from distinct steam explosion batches

**samples from the same pretreated batch

n.d. acronym for not detected (under the limit of detection of the device)

Ash is far more present in shives with a maximum of 11% for the untreated material. SE pretreatment seems to decrease its amount according to the severity factor applied to the biomass. In regards to the protein content, such proportions were expected as a significant amount of seeds was still present in the inflorescences. The pretreatment at higher temperature might have lowered the amount of detectable proteins by denaturing them or in case of by complexing with other molecules like cellulose and lignin as suggested by (Jaenicke, 1991 [66]). The proportion of soluble lignin present in shives increased, going from 2.0 % in untreated shives to 2.6 % (10 bar) and 2.4 % (20 bar) in pretreated shives. Its effect on lignin varies in regards to the inflorescences, since it went from 5.2% for untreated inflorescences to 3.4 % (10 bar) and 6.0 % (20 bar). The steam explosion might have different effects depending on the parameters applied (solubilization at a specific pressure/temperature, complexation with other components).

F**igure 11** provides an explicit illustration of the overall chemical composition of the biomasses. First of all, the characterization seems to be incomplete for some biomasses, with inflorescences having around 15% of their composition remaining undefined. However, these data needs to be read as minimum values. In order not to count the protein and the fat content which might have been extracted by water or by ethanol, those have been subtracted from the extractibles and are referred to as "other extractibles".

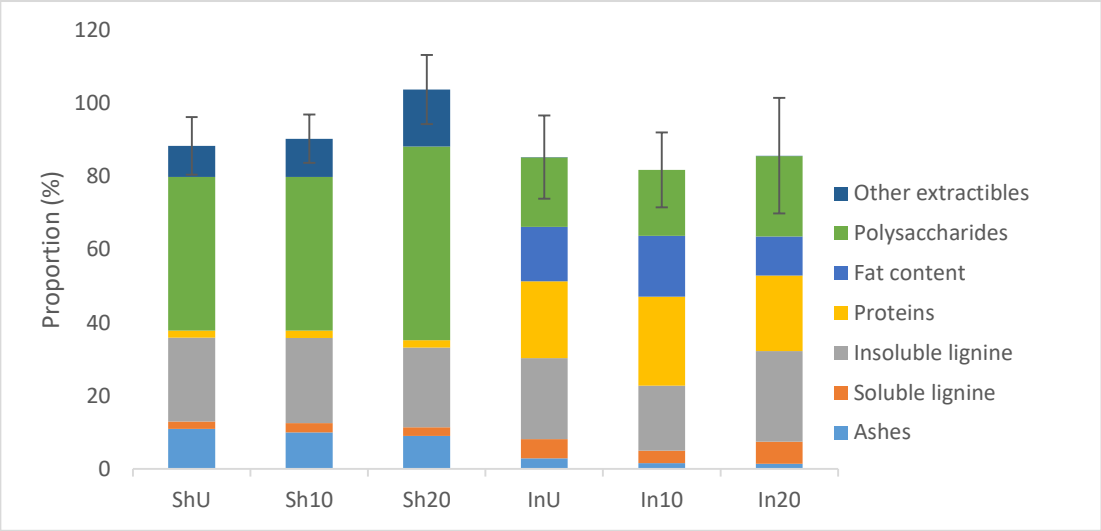

***Figure 11**: Composition of untreated shives (ShU) shives treated by steam explosion at 10 bar (Sh10) and 20 bar (Sh20), untreated inflorescences (InU) and inflorescences treated at 10 bar (In10) and 20 bar (In20).*

Inflorescences seem much more affected by the pretreatment than the shives considering the water extractibles as there is a decrease of about 12%. It can be assumed that the majority of these extractibles were evacuated in the effluents coming out of the steam explosion. Ethanol extractibles were not extracted by the steam/water in the reactor thus increasing their proportion in the biomass. Thus ethanol extractibles from the shives increased from 4.0 % (untreated) to 7.0 % (10 bar) and 10.2 % (20 bar) while those of inflorescences increased from 18.0 % (untreated) to 27.3 % (10 and 20 bar). The total amount of water and ethanol extractibles almost perfectly matched those of the protein and fat content which does not seem to be a coincidence. The amount of fat in shives was not investigated as it would not be significant.

Comparing the chemical and elemental composition of hemp shives and hemp inflorescences, the second appear to be a promising substrate for the production of enzymes in SSF considering the amount of proteins of more than 20%. This precious source of nitrogen might be useful for the growth of the microorganisms as well as the synthesis of enzymes. Hemp shives however, have a greater proportion in monosaccharides. Cellulose and hemicelluloses are of great interest in regards to the induction of enzymes. Therefore the shives may be some potential good candidates for further fermentations.

9.2 Elemental composition of the biomass

Elemental (C_{org}, N, P) compositions of the six biomasses were performed in order to acknowledge the steam explosion pretreatment effect on the raw materials and provide the essential raw data to understand its influence on solid-state fermentation in terms of microorganism development and enzymes productions.

Table 4 provides the elemental composition in organic carbon (C_{org}), nitrogen (N), and phosphorus (P) found in the biomass. The balance in these chemical elements is the most important since the study is about lignocellulosic materials.

***Table 4**: Elemental composition of the biomass on dry weight basis of untreated shives and inflorescences (ShU and InU) and pretreated by steam explosion at 10 and 20 bar (Sh10, Sh20, In10 and In20).*

Substrate	C_{org} (%)	N (%)	P (%)
ShU	44.8	0.3	0.03
Sh10	47.9	0.3	0.03
Sh20	50.4	0.3	0.02
InU	50.3	3.4	0.3
In10	53.2	3.9	0.3
In20	55.1	3.3	0.5

Strictly comparing the biomasses, the proportions of C_{org}, N and P found in shives are well under those of inflorescences. The highest percentages in C_{org} (55.1 %) and P (0.5 %) are found in In20 and the highest N (3.9 %) content is present in In10. The ratio in nitrogen and phosphorous for both materials is a factor of 10. An additional factor of 10 is noticeable between shives and inflorescences in those two chemical elements. The difference may probably come from the complexity of the inflorescences matrix which contains a significant amount of seeds (with a non-negligible content in fats and proteins). A difference of 5% in organic carbon seems to be steady regardless of the pretreatment.

STEX pretreatment had various effects on elemental composition with an apparent increase in organic carbon as the severity factor of the pretreatment increased for both biomasses. Having a carbon proportion of 44.8 % and 50.3 % in untreated shives and inflorescences, to 50.4 and 55.1 % once pretreated at 20 bar. Nitrogen and phosphorous content were steady for the shives, while phosphorous content in inflorescences clearly increased with an extraction at 20 bar, going from 0.3 to 0.5 %. However, the pretreatment effect on nitrogen is uncertain since

the nitrogen content went from 3.4 % (untreated), to 3.9% with an extraction at 10 bar and decreased with an extraction at 20 bar (3.3%).

9.3 Solid and liquid fractions: quantification of degradation products and fermentable monosaccharides

Since the biomass was pretreated under high conditions of pressure and temperature, the pretreatment must have an impact on the composition of the substrate. Free sugars were characterized on untreated and treated samples as well as in the residual water after the steam explosion. Degradation products (5-HMF, 2-F, lactic acid, formic acid, acetic acid and levulinic acid) formed during the steam explosion pretreatment were characterized in the solid and liquid fractions. The physico-chemical pretreatments are known to form enzymatic inhibitors, impairing the fermentation process when found in high concentration. Weak organic acids, phenolic compounds and furans may all have a negative effect on the fermentation [61]. These results are useful to understand the conversion of sugars present in the substrates after a harsh physicochemical pretreatment like the steam explosion. Furthermore the characterization will be more accurate and the enzymatic yields will be interpreted more completely.

Figure 12 shows the proportions of the three monosaccharides at the surface of the solid fraction and present in the liquid fraction. The concentrations for pretreated samples were estimated per 100g of solid fraction coming out of the steam explosion.

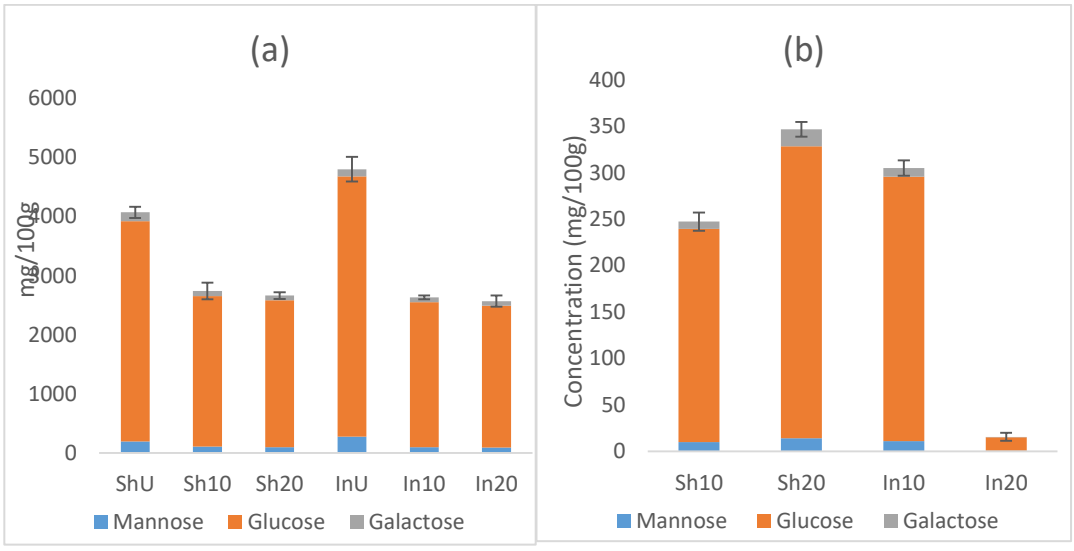

***Figure 12**: Free monosaccharides on untreated shives and inflorescences (ShU and InU) and pretreated by steam explosion at 10 and 20 bar (Sh10, Sh20, In10, In20) (a) in mg per 100 grams of solid substrate and*

(b) the respective washing water and effluents from the steam explosion in mg per 100 grams of collected substrate.

The highest concentration of free monosaccharides (4698 ± 209 mg/100g) was found on InU. According to the main component of the lignocellulosic biomass, glucose was the most present sugar. Surprisingly no xylose was found in either case. As expected, untreated samples had many more free monosacharides presents at the surface than steam exploded materials. Part of the sugars were washed away in the effluents and washing water as presented in (b). Sugars in washing waters from Sh20 were more concentrated than those of Sh10. Though the sugars concentration at the surface of the inflorescences are similar, those in the effluents are uneven as very few sugar was found in the effluents of In20.

Regarding the degradation products (**Table 5 and 6**), a wide range of molecules can be seen in various concentrations in regards to the fraction, the applied treatment and the biomass.

Table 5: *Degradation products in the solid fractions in mg per 100 g of biomass.*

	Sh10	Sh20	In10	In20
Lactic acid S	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Formic acid	0.0 ± 0.0	57.5 ± 16.3	0.0 ± 0.0	173.3 ± 7.2
Acetic acid	0.0 ± 0.0	596.2 ± 204.6	144.7 ± 19.2	0.0 ± 0.0
Levulinic acid	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	580.8 ± 95.1
HMF	1.2 ± 0.0	1.9 ± 0.3	1.1 ± 0.0	1.1 ± 0.1
2F	2.5 ± 1.0	2.3 ± 1.8	0.0 ± 0.0	0.1 ± 0.0

***Table 6**: Degradation products in the liquid fraction in mg per 100g of biomass recovered in the steam explosion tanker.*

	Sh10	Sh20	In10	In20
Lactic acid S	56.4 ± 3.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Formic acid	0.0 ± 0.0	20.1 ± 0.0	0.0 ± 0.0	1228.1 ± 349.9
Acetic acid	34.7 ± 8.8	1063.5 ± 21.1	0.0 ± 0.0	1569.3 ± 1166.0
Levulinic acid	0.0 ± 0.0	6464.9 ± 178.9	2008.4 ± 62.1	1400.9 ± 808.8
HMF	2.1 ± 0.0	22.4 ± 0.1	3.0 ± 2.2	2.6 ± 0.0
2F	0.2 ± 0.0	13.0 ± 1.2	0.0 ± 0.0	0.0 ± 0.0

Treatments at 20 bar (215°C) has way more impact on the formation of degradation products than the treatment at 10 bar (~200°C). The highest concentrations of acetic acid (1569 mg/100g) and formic acid (1228 mg/100g) was detected in the liquid fraction of In20. The highest levulinic acid concentration was present in the washing water of Sh20. Those three degradation products are predominant. Acetic acid is primarily derived from the deacylation of the xylan side chains of hemicellulose. No formic acid and few acetic acid (34 mg/100g) was detected at 10 bar because these products are typically formed by autohydrolysis pretreatment processes involving water at more than 200°C [61].

This is verified with the shives where the proportion in xylan diminished of around 1% between the untreated sample and the one at 20 bar. Acetic acid concentrations does not match perfectly with the characterization. 5-hydroxymethylfurfural and 2-furfural are both the first derivatives of glucose and xylose respectively and were present in very low concentrations. They are intermediate degradation products, forming formic acid and levulinic acid when subsequently rehydrated [62]. As expected with the conditions present in the reactor of the steam explosion (water impregnation and additional steam) and the washing, these two were found in larger concentrations than the intermediary products.

Most of the levulinic acid coming from the degradation of the shives at 20 bar (Sh20) was washed away by water. Since the washing process was absent for the inflorescences, much of the levulinic acid was present at the surface of the substrate after an extraction at 20 bar (215°C). In20 is a more complex matrix (flowers with many cannabinoids, seeds with protein, fatty acids) which might explain the diversity of products. Though much of the degradation

products derived from polysaccharides may have been quantified, many degradation products could not be determined and quantified (galacturonic acid, glycolic acid, humins and other acid-soluble compounds from the lignin degradation). The severity of steam explosion pretreatment had a huge impact on the degradation product. Consequently, part of the free sugar that was lost by the inflorescences between the untreated material and the one at 10 bar (~1900 mg/100g) was washed away by the washing water (300 mg/100g) and some was degraded into levulinc acid (~2000 mg/100g). Some of the sugar washed away from Sh10 was recovered in the effluents but around 75% (750 mg/100g) could not be determined as classical degradation product. Indeed one unidentified compound at a retention time of 14.8 min was eluated between the formic acid (RT=14 min) and acetic acid (RT=15.2 min). Though the peaks were close to each other, the three of them could distinctly be identified in Sh20 and being considered as different molecules and not as being a shift of the HPLC column.

10 Enzyme production

10.1 Experiment 1: Enzymatic production from *Sarocladium kiliense*

In the first experiment, *Sarocladium kiliense* was used as microorganism for the production of β-glucosidase and endoxylanase. Hemp shives (Sh) and hemp inflorescences (In) pretreated or not (ShU and InU) by steam explosion in two different conditions (10 and 20 bar) were tested as substrate in solid-state fermentation. Untreated wheat straw was used as reference substrate. Reactors conditions were duplicated and all measurements were performed in duplicates as well. Test for equal variances were done for each medium.

Cellobiase activity

The growth of *Sarocladium* in the different reactors was not constant during the 2 weeks of cultivation. Depending on the growing substrate the development seemed very unlike. For the first three days the microorganism didn't grow much. Spores were barely visible on hemp hurds and were completely absent on inflorescences. However, after 7 days of cultivation *Sarocladium* had colonized the entire area. At the end of the second week the microorganism was clearly visible in all flasks in different states of growth.

Figure 13 depicts the production of β-glucosidase after 7 and 14 days of cultivation.

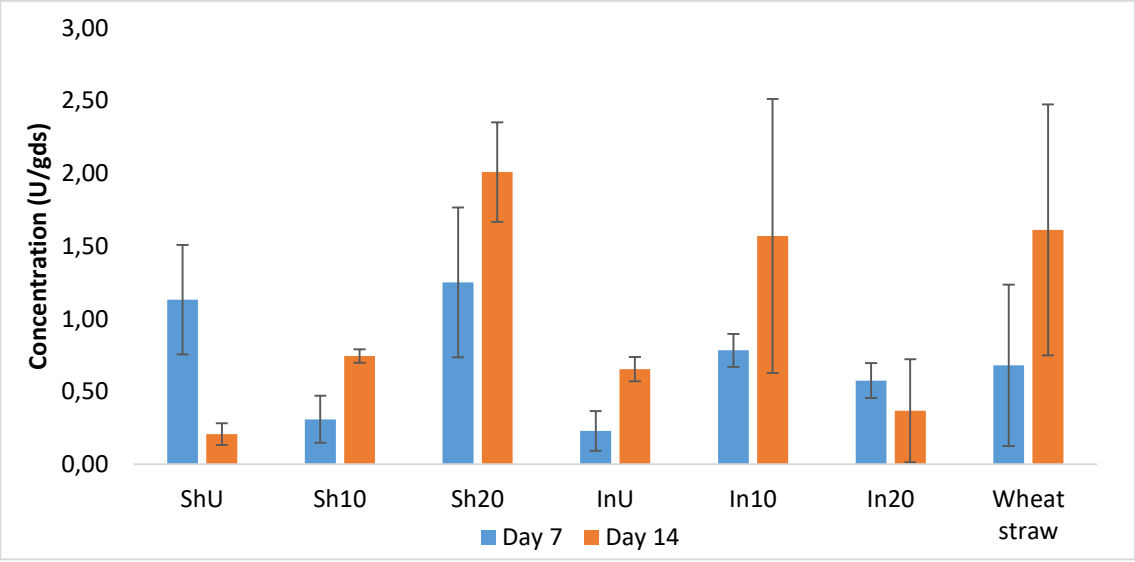

***Figure 13**: β-glucosidase activity in units per gram of dry substrate (U/gds) from* Sarocladium kiliense *after 7 and 14 days of SSF on untreated shives (ShU), shives pretreated at 10 and 20 bar (Sh10 and Sh20), untreated inflorescences (InU), inflorescences extracted at 10 and 20 bar (In 10 and In20) and wheat straw.*

After one week of fermentation, the highest cellobiase activity to be measured was on ShU and Sh20 with no significant differences ($p>0.05$) with the other substrates except Sh10 and InU.

Endoxylanase activity

The maximum enzyme activity (2.00±0.34 U/gds) was obtained on Sh20 after 14 days of fermentation but was not significantly different ($p>0.05$) from In10 (1.56±0.94 U/gds) and the activity on wheat straw (1.62 ± 0.86 U/gds). The three STEX pretreatment have a positive impact on the enzyme production (except on In20) and they are significantly different from each other ($p<0.05$) after 2 weeks of fermentation. Apparently the maximal β-glucosidase production by *Sarocladium* takes more than 2 weeks in these conditions to be released.
Figure 14 shows the production of endoxylanase by of *Sarocladium kiliense* after 7 and 14 days of cultivation.

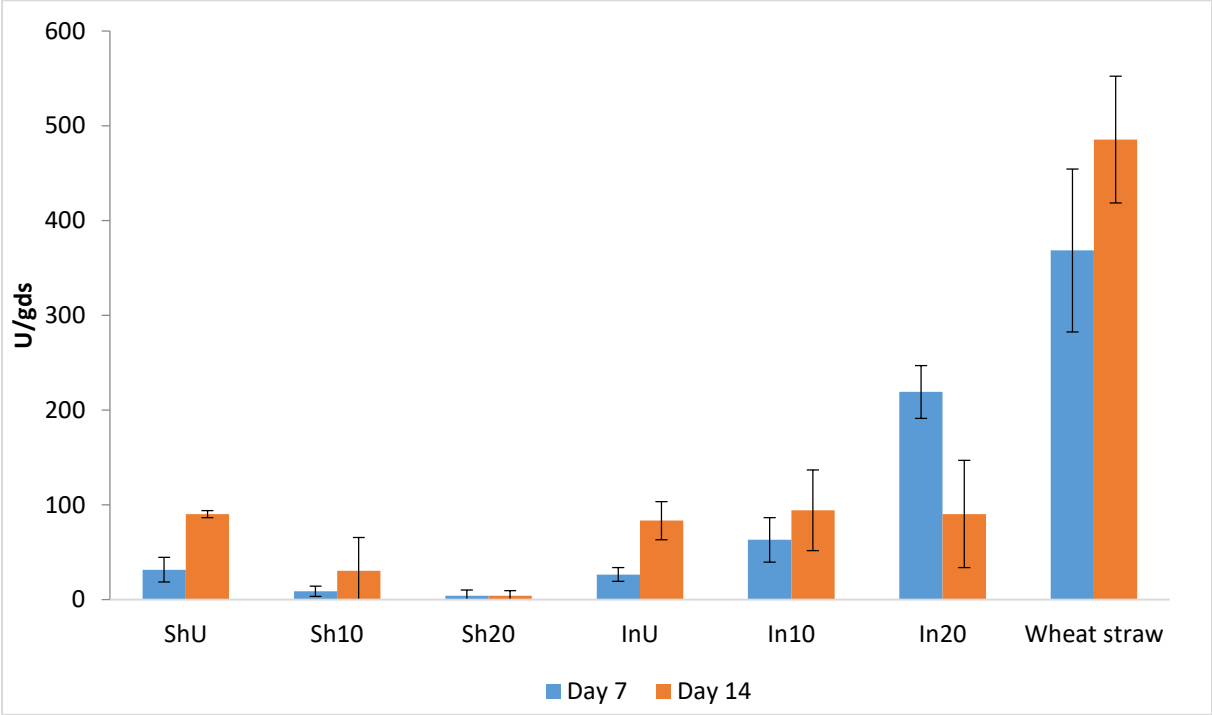

***Figure 14**: Endoxylanase activity in units per gram of dry substrate (U/gds) from* Sarocladium kiliense *after 7 and 14 days of SSF on untreated shives (ShU), shives pretreated at 10 and 20 bar (Sh10 and Sh20), untreated inflorescences (InU), inflorescences extracted at 10 and 20 bar (In 10 and In20) and wheat straw.*

The highest endoxylanase activity (485.6 ± 66.9 U/gds) was collected after 14 days of fermentation on wheat straw. Both productions are significantly very different ($p<0.01$) from those on shives and inflorescences. Maximal enzymatic production on hemp residues was on

In20 (219.18 ± 27.84 U/gds) after 7 days of fermentation. The least performant substrate was Sh20 (4.0 ± 5.0 U/gds) regardless of the time of cultivation. STEX pretreatments have a significant impact on the xylanase production ($p<0.05$), with a negative effect for the development of *Sarocladium* on shives and a positive one on the inflorescences. As for the cellobiase production, the microorganism seems to release a higher amount of enzymes after 2 weeks of fermentation.

Considering the enzymatic activity, *Sarocladium* seems to produce those enzymes with different trigger mechanisms because the enzymes yields are very different from each other and they do not follow a logical trend according to the substrate. In regards to the hemp by-products, shives are more likely to produce β-glucosidase and inflorescences had better results in xylanase productions. However wheat straw had a much more significant xylanase production than the hemp residues and its production in β-glucosidase was one of the highest after 14 days of culture. Having a look at the xylan content (which is a xylanase inducer) in the substrate reveals that wheat straw has the highest concentration of all substrates. The lower proportions in polysaccharides present in the inflorescences might be a limiting factor for the production of xylanases on shives and inflorescences.

Sarocladium (also known under the name *Acremonium*) has not been extensively studied in solid state fermentation. Yet several inhibitory products of cellulases produced by *Sarocladium* have been reported in case of a hydrothermal pretreatment [67]. The cellulase inhibitors were classified from the strongest to the weakest to be: xylo-oligomers > xylose > 2-F and 5-HMF > and finally acetic acid. However the acetic acid still present on Sh20 (596 mg/100g) had few inhibitory effects compared to the other substrate. Seeing the decrease of β-glucosidase activity from In10 to In20, we can assume that formic and levulinic acid may have an inhibitory effect on the enzyme production. Since the washing water of Sh20 contained a lot of levulinic acid, we can confirm the need of washing after a STEX pretreatment at more than 200 °C. As state by [68, 69], the above yields in β-glucosidase activity collected from *Sarocladium* was very low compared to other studies.

Regarding the xylanase productions on shives, the STEX pretreatment had a negative outcome. Acetic acid might completely inhibit the xylanases synthesis from *Sarocladium* at a specific concentration since its high amount present at the surface of Sh20 opposes its poor yields in enzyme. In comparison to the study of other microorganisms in SSF culture, the xylanase activity on wheat straw is not among the worse compared to other studies. [70]

10.2 Experiment 2: Enzymatic production from *Penicillium canescens*

Cellobiase activity

Penicillium canescens was used as enzyme producer during the second experiment. This soil fungus grows particularly well on dead plant materials thus being a good choice of microorganism for the experiment. **Figure 15** shows the β-glucosidase activities on the seven substrates.

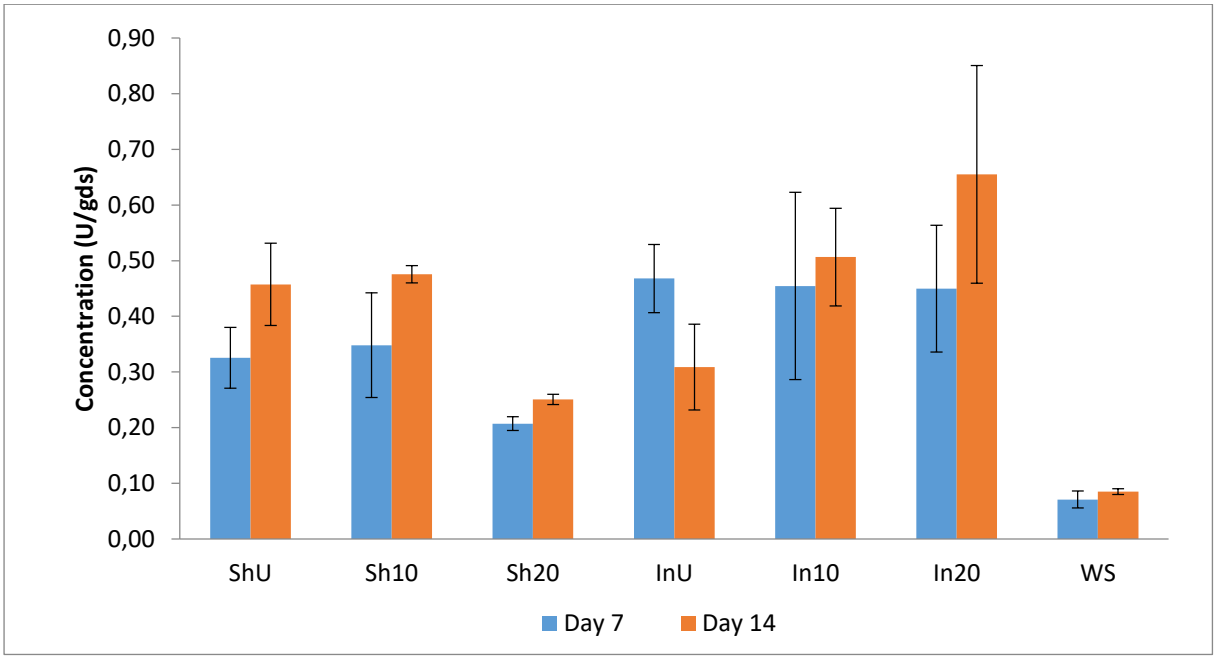

***Figure 15**: Cellobiase production in units per gram of dry substrate (U/gds) from* Penicillium canescens *after 7 and 14 days of SSF on untreated shives (ShU), shives pretreated at 10 and 20 bar (Sh10 and Sh20), untreated inflorescences (InU), inflorescences extracted at 10 and 20 bar (In 10 and In20) and wheat straw.*

The maximum β-glucosidase activity (0.65 ± 0.19 U/gds) produced by *Penicillium* was collected on In20 after 14 days of fermentation. The mean wasn't significantly different from In10 and Sh10 at the time of the enzyme collect. Strangely *Penicillium* didn't produce a lot of β-glucosidase on wheat straw. It was the least performant (0.07 ± 0.02 U/gds) substrate in regards to the cellobiase activity.

After 7 days of cultivation the enzyme activities between untreated and treated materials are not significantly different ($p<0.05$). However, the difference is significantly higher after 14 days of culture for the pretreated inflorescences ($p<0.05$). Two weeks of fermentation for *Penicillium*

seems more suited for the β-glucosidase production since almost all biomass have higher means, except InU.

Even though the different biomass have an unequal glucan content (32% for shives, 11% for inflorescences and 26% for wheat straw), respective β-glucosidase activities cannot be related to the proportions.

Endoxylanase activity

Figure 16 depicts the enzymatic production from *Penicillium canescens* after 7 and 14 days of cultivation.

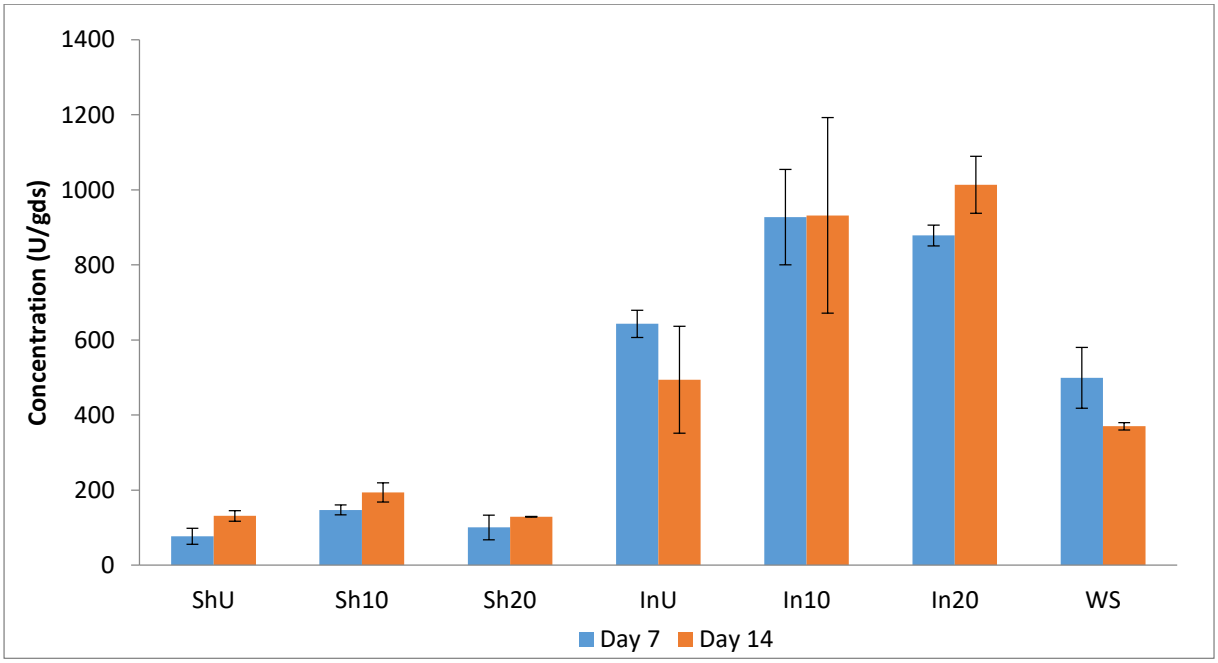

***Figure 16**: Endoxylanase activity in units per gram of dry substrate (U/gds) from* Penicillium canescens *after 7 and 14 days of SSF on untreated shives (ShU), shives pretreated at 10 and 20 bar (Sh10 and Sh20), untreated inflorescences (InU), inflorescences extracted at 10 and 20 bar (In 10 and In20) and wheat straw.*

The highest endoxylanase activity was measured on In20 (1013.3 ± 75.8 U/gds) after 2 weeks of fermentation. All enzymatic productions on treated inflorescences are significantly higher ($p<0.05$) than on other substrates. The substrate with the worst activity was ShU after 7 days of fermentation (77.2 ± 21.4 U/gds).

Variance analysis reveals a significant difference ($p<0.05$) of production according to the treatment applied on the substrate and a clear trend can be determined between substrates used. The xylanase production slightly increased between the two weeks of fermentation but the difference wasn't significant ($p<0.05$). The control substrate has an intermediate

production between hemp inflorescences (most performant) and hemp hurds which didn't show much activity.

The chemical composition of the substrates had a clear impact considering the general productions of xylanases by *Penicillium*. The first parameter that may have an influence on the enzyme production was the C:N ratio of the substrates. This ratio was of 15:1 for inflorescences while the ratio on hemp shives was of 140:1 which is a completely unbalanced ratio as plant material. For example, a classical straw residue ratio is about 70:1 and humus mineral soil is around 10:1. It is reported in [71] that at a ratio of 16:1 all the nitrogen in the medium is used by fungi for the synthesis of its cells. A specific C:N ratio would be required by fungi for biochemical syntheses. A medium with a ratio between 10:1 and 12:1 is considered as adequate for growth and for synthesis of nitrogen-containing by-products. Since enzymes are proteins containing nitrogen, this is typically the by-product concerned. With almost 20% of protein and thus a more balanced C:N ratio the inflorescences are more likely to host fungi as a substrate for SSF.

Penicillium canescens vs *Sarocladium kiliense*

Apparently *S.kiliense* and *P.canescens* produced enzymes in different concentrations in regards to their cellobiase and endoxylanase activities, even if all fermentation conditions were kept the same. The big trend is, on the one hand, a better production of β-glucosidase by *Sarocladium*, with a maximum yield of more than 3 times the one of *Penicillium*. On the other hand, *Penicillium* has a maximal xylanase production more than 2 times higher than *Sarocladium*, and almost 5 times when only hemp residues are considered. The fermentations on hemp shives were only profitable in regards to their β-glucosidase yields when compared to the inflorescences. The fermentation on wheat straw is the most profitable when using *Sarocladium*.

Considering the objective to be the valorization of hemp byproducts, the best candidate for an overall valorization seems to be *Penicillium*. This is why the microorganism was selected for a third experiment with improved fermentation conditions.

10.3 Experiment 3: Improvement on selected parameters

In the third experiment, a few parameters were considered to be optimized in order to increase the enzymatic productions. The hypotheses were:

1) That the maximal enzyme production coming from *Penicillium* growing on inflorescences wasn't reached after two weeks of fermentation
2) That the C:N ratio was unbalanced in hemp shives and couldn't sustain the growth of the microorganism and/or the synthesis of the enzymes
3) *Penicillium* was not in close contact with hemp shives because of the raw morphology of the chips.

Moreover, an additional experiment was carried out on inflorescences. According to Capna Labs (California), the most efficient way to extract cannabidiol from the flowers is considered to be the ethanol extraction. Therefore a SSF substrate based on flowers extracted residues was considered. **Figure 17** shows the state of the fermentation after 7 days of culture on ethanol extracted inflorescences (a) and on inflorescences extracted by STEX at 20 bar (b).

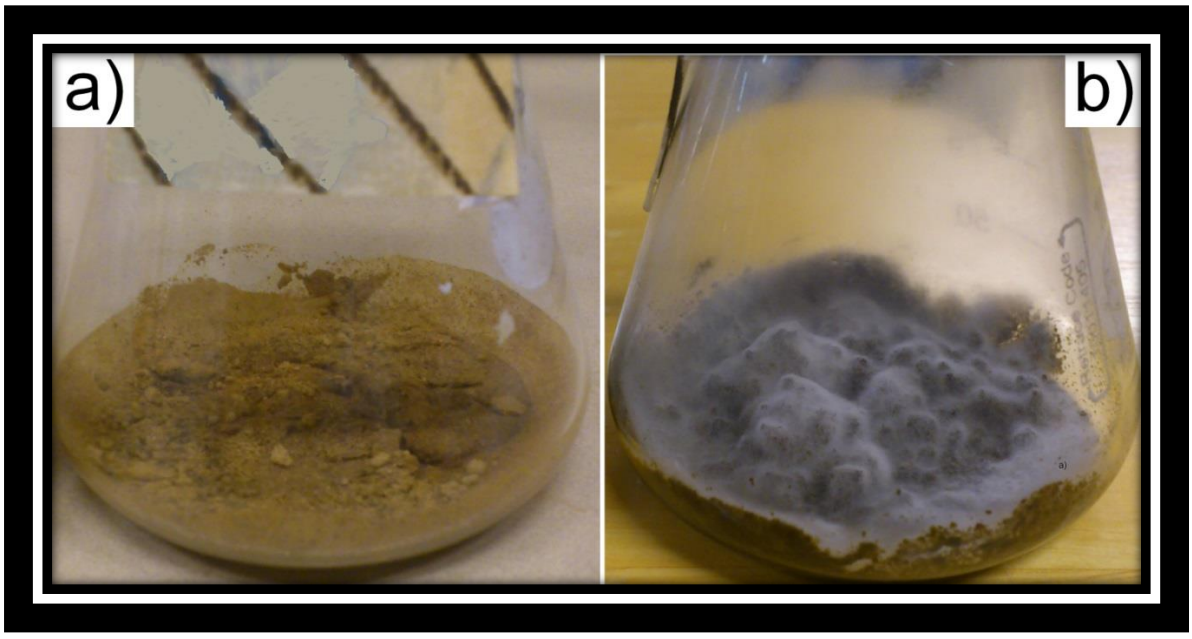

Figure 17: Penicillium *cultivation on ethanol extracted flowers (a) and flowers extracted by steam explosion (b) (20 bar, 215°C) after 7 days of fermentation.*

Even though the residual ethanol was completely removed from the substrate by evaporation for 48h, *Penicillium* didn't grow on the substrate at all. Picture b) shows a classical outlook of the microorganism's growth. The substrate had either all the needed nutrients removed by the

extraction, or more probably a small amount of ethanol was still present on the substrate and could not be efficiently removed. Many scientific papers have reported the effect of ethanol on fungal cultures. Growth of spores from *Penicillium sp.* is generally inhibited at concentration as low as 5 to 8 % (v/v). The minimum ethanol concentration for the germination of spores was also estimated to be between 2 and 6 % for some species of fungi [67]. An additional hypothesis is a possible inhibitor generated during the ethanol extraction. Whether the substrate was depleted in nutrients or the ethanol concentration was still too high, the biomass is not worth the use after this extraction method.

Part of the third fermentation involved the culture of *P. canescens* in SSF on inflorescences for 3 weeks. The fermentation parameters were kept the same as in experiments 1 and 2. **Figure 18** depicts the results of the β-glucosidase and xylanase productions after 21 days of fermentation by *Penicillium*. Enzymes yields after 7 and 14 days are shown in parallel for an easy comparison.

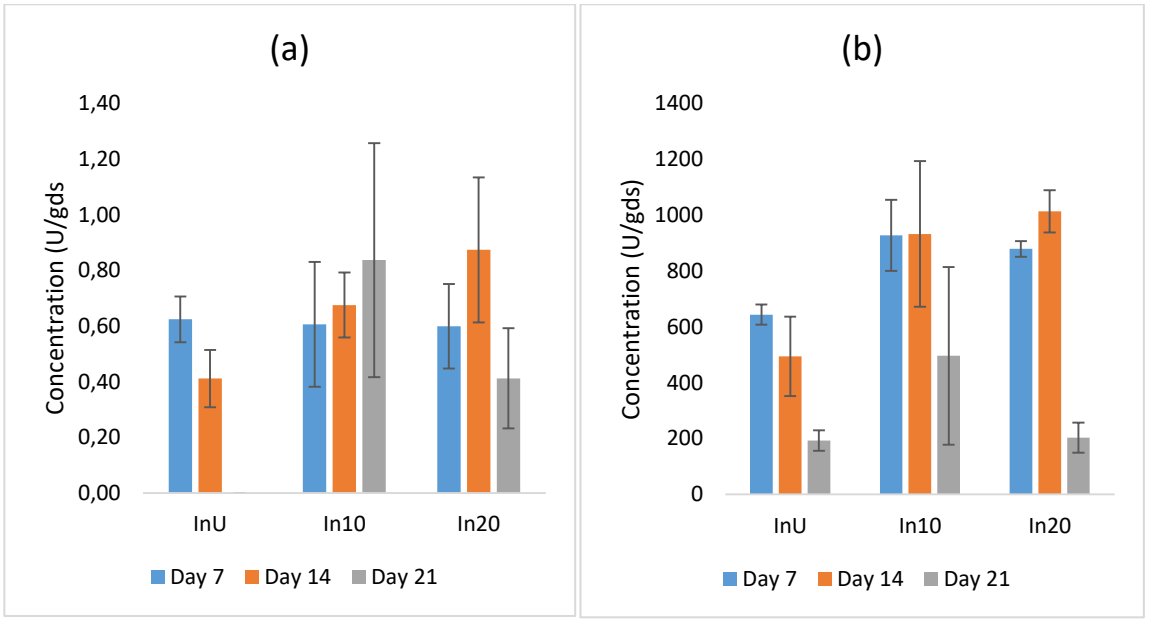

***Figure 18**: Cellobiase (a) and endoxylanase (b) activities from* Penicillium canescens *after 7, 14 and 21 days of fermentation on untreated inflorescences (InU) and pretreated inflorescences at 10 and 20 bar (In10 and In20).*

The β-glucosidase production didn't show a significant improvement after 21 days of fermentation. The highest production was collected on In10 with 0.84 ± 0.42 U/gds but wasn't significantly different from the production on In20 (0.87 ± 0.26 U/gds). InU did not produce any measurable cellobiase activity and In20 productions were lower than after 14 days. The graph of the xylanase productions has a similar look than the cellobiase with two low endoxylanase activities by InU and In20 and a higher production of 496 ± 318 U/gds by In10.

21 days of fermentation was barely profitable in regards to the enzymatic productions of *Penicillium*. Since the C:N ratio of hemp shives was completely unbalanced for the growth of fungi, additional nutrient was added to the fermentation. Based on the former nutrients (sole N source was yeast peptone and yeast extract) and the hemp shives composition, it was determined that 0.1g of N had to be added in each reactor in the form of $(NH_4)_2SO_4$ .The effect of nitrogen and milling on the xylanase production on hemp shives is depicted in the **figure 19.**

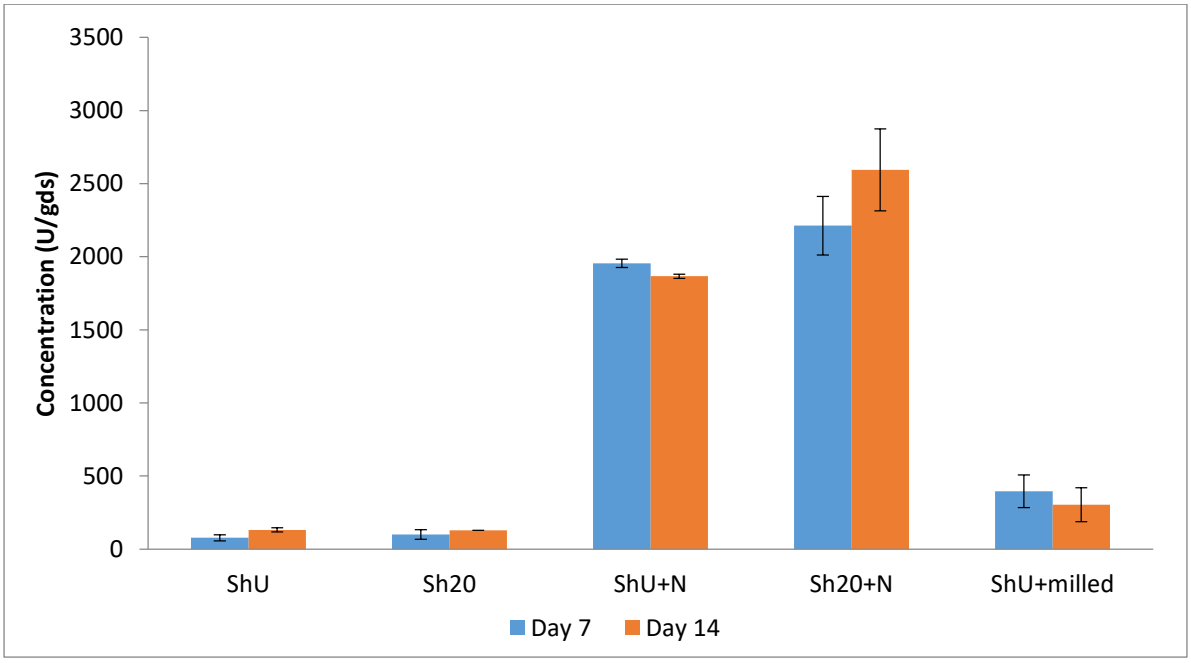

***Figure 19**: Xylanase activities in units per gram of dry substrate (U/gds) from* Penicillium canescens *on untreated shives and pretreated shives at 20 bar with (ShU+N and Sh20+N) and without (ShU and Sh20) additional nitrogen in the nutrients or milled shives (ShU milled).*

The production of xylanase by *Penicillium* has greatly improved with the nutrient correction. After the nitrogen supplementation, the enzyme activity collected after 14 days of fermentations on ShU went from 131 U/gds to 1867 U/gds. The productions on treated shives had even better results, going from 129 U/gds to 2595 U/gds. The difference was significantly higher ($p< 0.05$) than all the other yields. The yields on milled shives increased to 304-396 U/gds. The experiment validated the previous hypothesis of a lack of nitrogen limiting the enzymatic production from *Penicillium canescens*. **Figure 20** shows the yields in β-glucosidases that were obtained from the same fungus on ShU and Sh20 after 7 and 14 days of fermentation with optimized parameters.

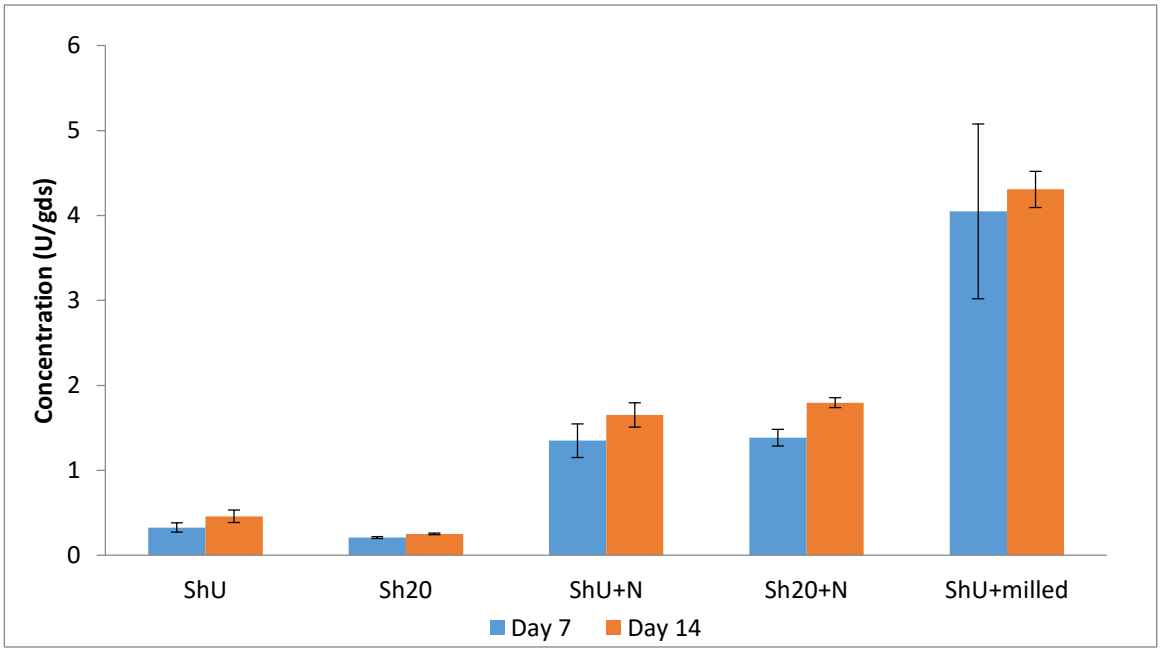

***Figure 20**: β-glucosidase activities in units per gram of dry substrate (U/gds) from* Penicillium canescens *on untreated shives and pretreated shives at 20 bar with (ShU+N and Sh20+N) and without (ShU and Sh20) additional nitrogen in the nutrients or milled shives (ShU+milled)*

The β-glucosidase productions had different outcome than the xylanase productions in regards to the modified parameters. The highest cellobiase activity was collected on milled shives with an average of 4.3 U/gds. The additional nitrogen had a positive effect on the *Penicillium*, ranging from 1.3 U/gds to 1.80 U/gds. Variance analysis indicates that untreated hemp shives, hemp shives with additional nitrogen and milled hemp shives are all significantly different from each other ($p<0.05$). However, the STEX pretreatments didn't have a significant impact ($p<0.05$) on the β-glucosidase production. Like the xylanase production, the cellobiase production increased significantly by sole addition of nitrogen which once again confirmed the assumption that the unbalanced C:N ratio of shives had to be corrected. Moreover, a modification of the morphology of the substrate showed an important increase in β-glucosidase. Even though the raw material was chopped in little pieces, *Penicillium* probably needed a closer contact with the shives in order to produce some cellobiase activity. The milling of the substrate was the best way for the fungi to be in contact of cellobiase-inducing molecules such as cellobiose [73].

Experiment 3 highlights a peak of enzyme production probably after two week of fermentation, a need for nitrogen supplementation for the xylanase excretion by *Penicillium* on hemp shives and the crucial role of the granulometry of the substrate in the excretion of β-glucosidase.

The last experiment was conducted for 3 days. ShU and Sh20 were milled and used as SSF substrate with additional nitrogen in the nutrients. **Figure 21** shows the results in xylanase productions in comparison to the production on shives without milling from experiment 3. The β-glucosidase activity was completely missing from the batch n°4.

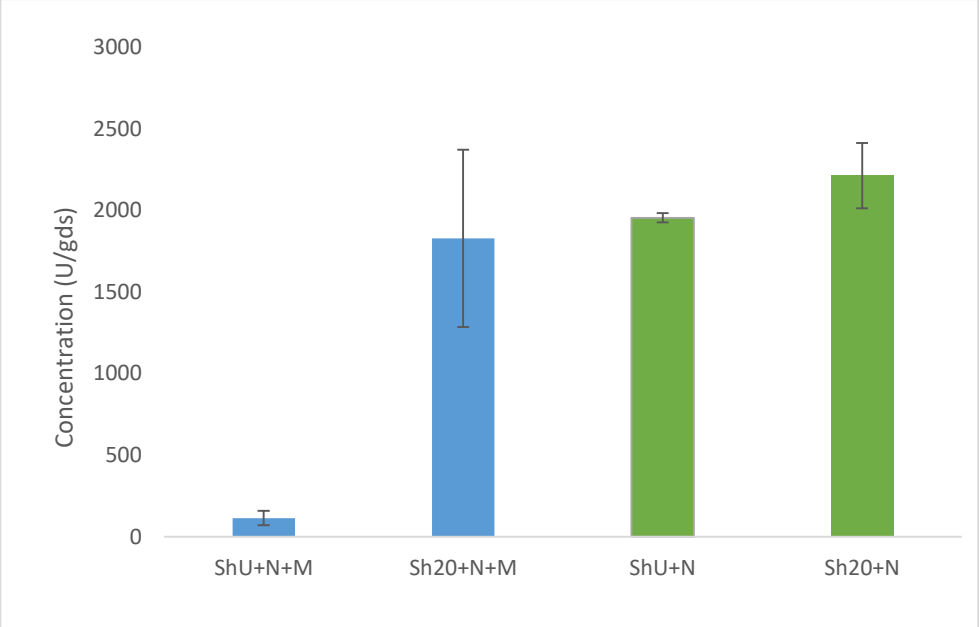

***Figure 21**: Xylanase activity by* Penicillium canescens *after 3 days of SSF on untreated and pretreated milled hemp shives with additional nitrogen in the nutrients (ShU+M+N and Sh20+N+M) and shives untreated and pretreated at 20 bar with additional nitrogen in the nutrients (ShU+N and Sh20+N) after 7 days of fermentation.*

After 3 days of fermentation, the highest xylanase activity was obtain on shives pretreated by steam explosion at 20 bar (1828 ± 542 U/gds). Untreated substrate only produced 114 ± 44 U/gds. The difference between untreated and pretreated material is striking at this stage of the fermentation. Most probably, the production of xylanase by *Penicillium* was delayed on untreated shives. Either the fungi had more difficulties to settle than on steam exploded shives, or the steam explosion and milling pretreatment highly increased the proximity of the fungi with inducing molecules such as xylan. Another possibility is a possible delay coming from the presence of xylanase-repressing molecules in higher amount on untreated material (mainly monosaccharides). In comparison, the highest production after 7 days of fermentation without the milling pretreatment on Sh20 reached 2213 ± 201 U/gds. Considering the probability that *Penicillium* did not reach its highest production after 3 days (rather around 14 as experiment 2

suggest), it is safe to assume even higher yields once the shives are milled and supplemented with a more significant amount of nitrogen.

11 Discussion on induction and repression of the catabolic system

The enzymatic production of filamentous fungi is deeply regulated by catabolic induction/repression processes. Throughout the study, the synthesis and secretion of the two enzymes were mainly dependent of the substrate on which the fungus had grown on.

The STEX pretreatment modified the substrate at a morphological and chemical level. It had a noticeable influence on the productions on inflorescences as well as on the xylanase productions when supplied in nitrogen. Some parameters such as free monosaccharides (mainly glucose) have been reported to cause a repression of cellulase and xylanase synthesis as well as partial inactivation of enzymes [74]. The reason why there is such a big difference in xylanase productions untreated and treated material could partially come from the difference of free glucose available on the substrates. Other catabolic repression might come from the crystallinity of the cellulosic material, which has also shown an impact on both enzymes expression [74]. Since thermochemical pretreatment decreases the degree of polymerization, increase the crystallinity of the substrate and opening the structure (Jacquet et al[23]) this is another possible effect that favors the induction of (hemi)cellulolytic enzymes.

As the yields were compared to other studies, some numbers were striking. The yields in both xylanases and β-glucosidase widely vary in regards to the *Penicillium* species and the culture conditions but in general, the β-glucosidase productions were generally much higher (up to 150 U/gds[70])than the present yields (maximum 4.35 U/gds). Some numbers in xylanase productions were well under those found in this study with maximums around 600 U/gds for *Penicillium echinulatum* [70] but as they were compared to the articles concerning *Penicillium canescens*, those numbers in xylanase activities were much higher (from 7448 U/g on wheat straw [59] to up to 14485 U/g on soya oil cake [75]). Some clues were given in the article, pointing out the fact that the strain *Penicillium canescens* had been previously selected in laboratory and designed for not producing a large amount of cellulase. This may explain the low yields in β-glucosidase.

More than substrate, the nutrients and particularly mineral salts provided to the microorganism are also very important. Mineral additives have a huge effect on xylanase activity on this strain of *Penicillium* [75]. Among the mineral salts provided by the nutrients, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$ were reported as deleterious for the xylanase production.

Other tracks may explain the difficulties encounter in the experiment explaining the low xylanase activity. Looking more precisely at the enzyme itself, another key player in the efficiency of the activity is the carbohydrate-binding modules (CBMs), an important amino-acid sequence helping the enzyme to attach the substrate. Its degradation or even of the catalytic module by some proteases released during the fermentation process may inactivate the enzyme[76]. Other key parameters that may have influence the enzymatic production is the pH in the reactor, the extraction process of the enzyme and the moisture of the reactor. The first can only be controlled at the beginning of the SSF and has an important role in the optimal growth of the microorganism as well as the xylanase productions. The second is important to completely detach the enzyme from the substrate and the current shaking (80 rpm) may be too low. A more virulent shaking may have benefited the recovery of the enzyme. And finally the moisture content in the reactor which is not easy to control during the fermentation process since it depends on the physical properties of the substrate and the growth of the microorganism.

V. Conclusion

In conclusion, hemp shives and hemp inflorescences were investigated as substrate for the production of β-glucosidase and xylanase in solid-state fermentation using *S.kiliense* and *P.canescens*.

The pretreatment by steam explosion at 20 bar was the most efficient in regards to enzyme productions. Experiments highlighted a poor proportion of polysaccharides in the inflorescences (19.2 ± 2.5 %) which is undesirable for the induction of enzymes and a lack of nitrogen in the shives (unbalanced C:N ratio of 140:1). The supplementation of nitrogen showed a huge increase in xylanase productions with a maximum of 2595 ± 280 U/gds on pretreated shives (20 bar) by *Penicillium*. The enzyme productions from *Sarocladium* were much lower. The granulometry of the substrate played a significant role in the enzyme production since the highest β-glucosidase productions (4.05 ± 1.03 U/gds) were reached on milled shives. Steam explosion pretreatment, milling process and supplementation of nitrogen might very well be the three key parameters acting in synergism for solid-state fermentation of hemp shives. 14 days seems to be the most suited amount of time for fermentation for both fungi. *Penicillium canescens* seemed to be designed for xylanase production and is barely profitable in regards to β-glucosidase.

The steam explosion distinctively enhanced the enzymatic productions but considering the poor biomass recovery of the inflorescences (10%), the process is only profitable with the shives (70%). Higher productions might be achieved with more drastic steam explosion conditions (for example increasing the pressure up to 40 bar). In order to avoid catabolic inhibitors such as organic acids, the substrate need to be washed with water. The nutrients provided to the fungi need to be carefully controlled. The xylanase production is highly dependent on the minerals additives and it is crucial avoid those that have an inhibitory effect.
In regards to commercial applications, shives are possible cheap alternatives to purified xylan for reducing the cost of the enzyme production. In an industrial perspective, further optimizations need to be performed before the consideration of a scale-up of the reactor.

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VII. Appendix

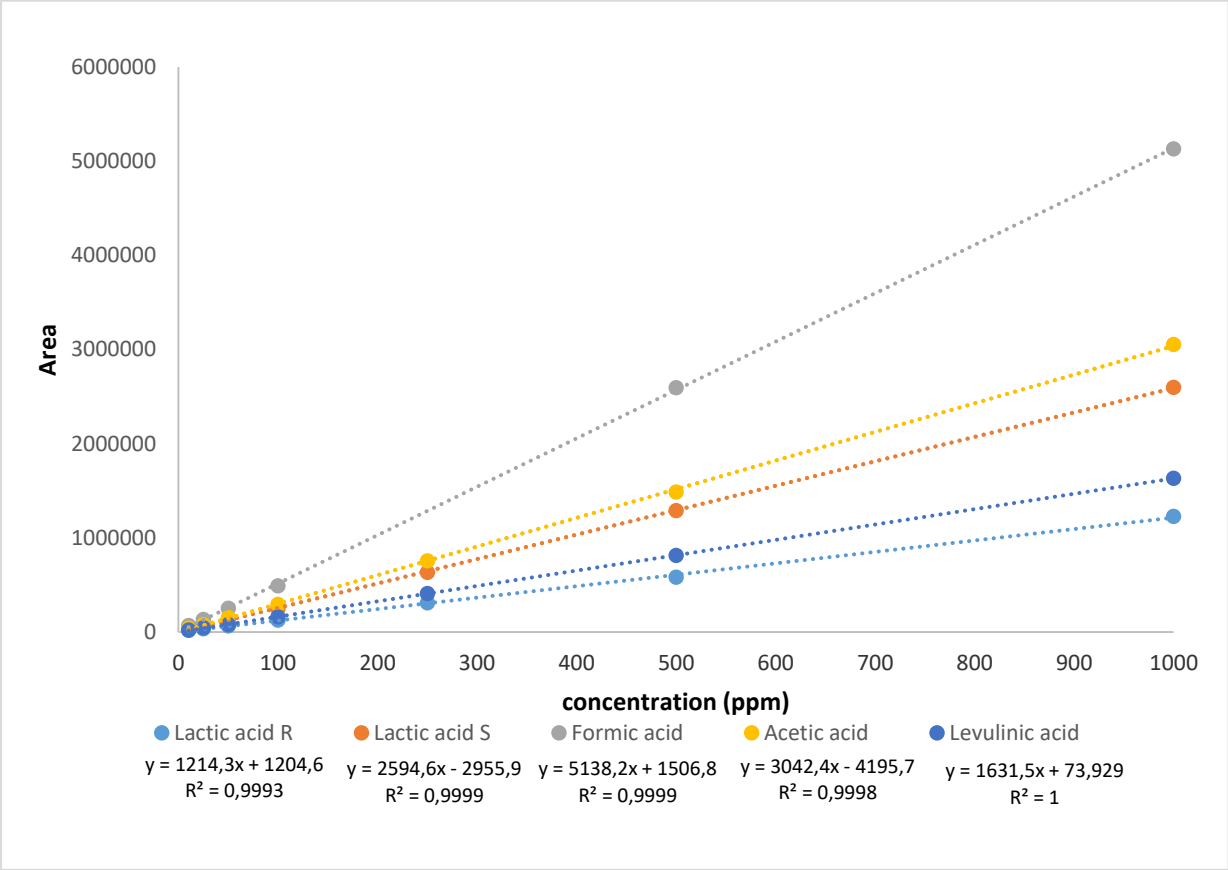

***Figure 22**: Calibration curve of acid degradation products.*

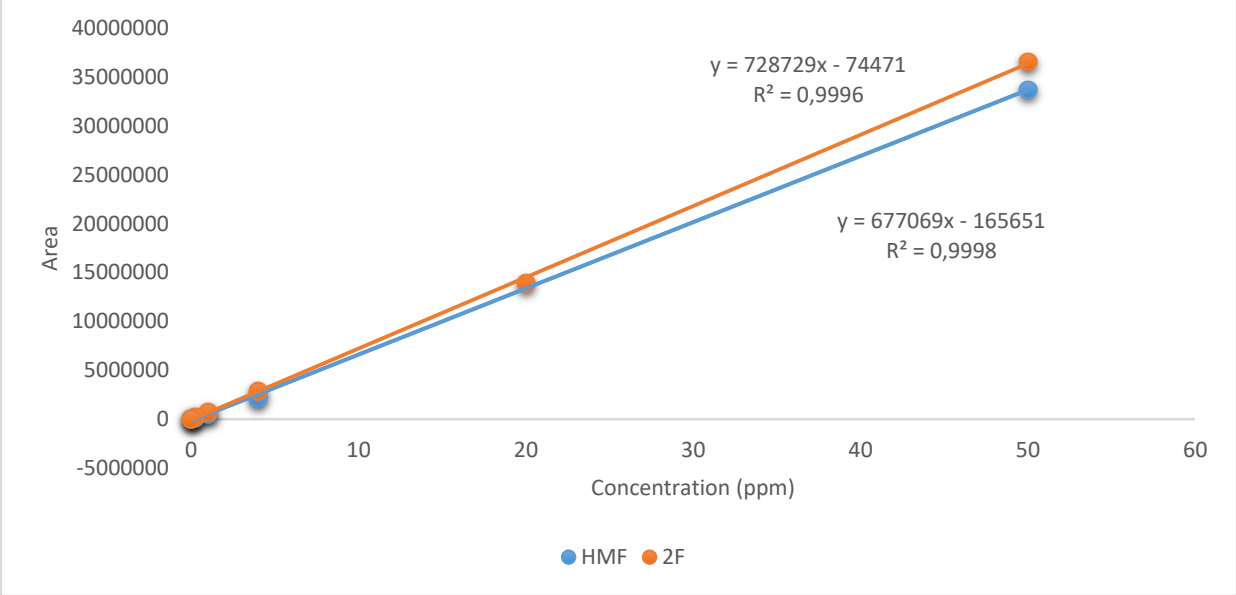

***Figure 23**: Calibration curve of 5-HMF and 2-F.*

***Table 7**: SSF conditions for the first experiment with* Sarocladium kiliense.

Substrate	Treatment	Days of fermentation	Repetitions	Nutrients
Hemp shives	none	7	2	1X
Hemp shives	none	14	2	1X
Hemp shives	10 bar	7	2	1X
Hemp shives	10 bar	14	2	1X
Hemp shives	20 bar	7	2	1X
Hemp shives	20 bar	14	2	1X
Inflorescences	none	7	2	3X
Inflorescences	none	14	2	3X
Inflorescences	10 bar	7	2	3X
Inflorescences	10 bar	14	2	3X
Inflorescences	20 bar	7	2	3X
Inflorescences	20 bar	14	2	3X

***Table 8**: SSF conditions for the second experiment with* Penicillium canescens.

Substrate	Treatment	Days of fermentation	Repetitions	Nutrients
Hemp shives	none	7	2	1X
Hemp shives	none	14	2	1X
Hemp shives	10 bar	7	2	1X
Hemp shives	10 bar	14	2	1X
Hemp shives	20 bar	7	2	1X
Hemp shives	20 bar	14	2	1X
Inflorescences	none	7	2	3X
Inflorescences	none	14	2	3X
Inflorescences	10 bar	7	2	3X
Inflorescences	10 bar	14	2	3X
Inflorescences	20 bar	7	2	3X
Inflorescences	20 bar	14	2	3X

***Table 9**: SSF conditions for the third and fourth experiment with* Penicillium canescens.

Substrate	Treatment	Days of fermentation	Repetitions	Nutrients	Particular treatment
Wheat Straw	none	7	2	1X	
Wheat Straw	none	14	2	1X	
Hemp shives	none	7	2	1X+N	
Hemp shives	none	14	2	1X+N	
Hemp shives	20 bar	7	2	1X+N	
Hemp shives	20 bar	14	2	1X+N	
Hemp shives	none	7	2	3X	milled
Hemp shives	none	14	2	3X	milled
Inflorescences	none	7	2	3X	Ethanol extraction
Inflorescences	none	21	2	3X	
Inflorescences	10 bar	21	2	3X	
Inflorescences	20 bar	21	2	3X	
Hemp shives	none	3	3	3X+N	milled
Hemp shives	20 bar	3	3	3X+N	milled
Nutrients	none	3	1	3X+N	

***Table 10**: Endoxylanase production by* Sarocladium *(U/gds) during the first experiment.*

Substrate	Average day 7	Standard deviation day 7	Average day 14	Standard deviation day 14
ShU	32	13	90	4
Sh10	9	5	31	35
Sh20	4	6	4	6
InU	26	7	83	20
In10	63	24	94	43
In20	219	28	90	57
Wheat straw	368	86	485	67

***Table 11**: Cellobiase productions by* Sarocladium *(U/gds) during the first experiment.*

Substrate	Average day 7	Standard deviation day 7	Average day 14	Standard deviation day 14
ShU	1.13	0.38	0.21	0.07
Sh10	0.31	0.16	0.74	0.05
Sh20	1.25	0.51	2.01	0.34
InU	0.23	0.14	0.65	0.08
In10	0.78	0.11	1.57	0.94
In20	0.57	0.12	0.37	0.35
Wheat straw	0.68	0.55	1.61	0.86

***Table 12**: Endoxylanase production by* Penicillium *(U/gds) during the second experiment.*

Substrate	Average day 7	Standard deviation day 7	Average day 14	Standard deviation day 14
ShU	77	21	131	14
Sh10	147	13	194	26
Sh20	100	33	129	1
InU	643	36	494	142
In10	927	127	932	261
In20	878	28	1013	76
WS	499	81	370	10

***Table 13**: Cellobiase productions by* Penicillium *(U/gds) during the second experiment.*

Substrate	Average day 7	Standard deviation day 7	Average day 14	Standard deviation day 14
ShU	0.33	0.05	0.46	0.07
Sh10	0.35	0.09	0.48	0.02
Sh20	0.21	0.01	0.25	0.01

InU	0.47	0.06	0.31	0.08
In10	0.45	0.17	0.51	0.09
In20	0.45	0.11	0.65	0.20
WS	0.07	0.02	0.09	0.01

***Table 14**: Cellobiase production by* Penicillium *(U/gds) during the third experiment.*

	Average day 7 or 21	**Standard deviation day 7 or 21**	**Average day 14**	**Standard deviation day 14**
ShU+N	1.35	0.20	1.65	0.14
Sh20+N	1.38	0.10	1.80	0.06
ShU+milled	4.05	1.03	4.31	0.21
InU 21 days	-0.36	-0.14		
In10 21 days	0.56	0.41		
In20 21 days	0.14	0.20		

***Table 15**: Endoxylanase production by Penicillium on shives (U/gds) during the third experiment.*

	Average day 7 or 21	**Standard deviation day 7 or 21**	**Average day 14**	**Standard deviation day 14**
ShU+N	1954	28	1867	14
Sh20+N	2213	201	2595	280
ShU milled	396	112	304	116
InU 21 days	192	36		
In10 21 days	496	317		
In20 21 days	202	53		