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Master's Thesis

Assessment of the enzymatic saccharification of poly-saccharides contained in spent mushroom substrate

Master's in applied and Commercial Biotechnology

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Ayesha Liaqat

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Abbreviations

AAH	Analytical acid hydrolysis
AES	Analytical enzymatic saccharification
CBD	Carbohydrate-binding domain
CBH	Cellobiohydrolases
CMC	Carboxy methyl cellulase
DNS	Dinitro salicylic acid
DW	Dry weight
FPA	Filter paper activity
HPLC	High-performance liquid chromatography
HTP	Hydrothermal pretreatment
PES	Preparative enzymatic saccharification
SMS	Spent mushroom substrate

Table of Contents

List of Tables

List of Figures

Abstract

Spent mushroom substrate (SMS) is a leftover lignocellulosic biomass from mushroom cultivation that is rich in cellulose, hemicelluloses, lignin, and other biological components, making it a potential renewable resource for bioconversion processes for producing biofuels and other bio-based products. In this study, the enzymatic saccharification of SMS from two commercially important mushrooms, shiitake (*Lentinula edodes*) and oyster (*Pleurotus ostreatus*), cultivated on conventional (birch or oak sawdust) and non-conventional (spelt, wheat, oat straw, and coffee chaff) initial substrates, was investigated. The non-conventional oyster SMS is characterized by higher cellulose (up to 41.8% (w/w) in oat straw), lignin (32.6 % in wheat straw), water extractives (22.5% in oat straw), ethanol extractives (5.4% in the SMS containing coffee chaff), and ash content (13.8% in oat straw) compared to conventional SMS. For enzymatic saccharification, two commercial enzymes (Cellic CTec2, *Trichoderma* cellulases) and one experimental enzyme (ExpC) were used. Using Cellic CTec2, the analytical enzymatic saccharification of shiitake SMS resulted in digestibility of cellulose and xylan over 90 and 70% (w/w), respectively). An improved digestibility was observed when the extractive compounds were removed. The enzymatic digestibility of oyster mushroom SMS was lower than that of shiitake SMS. Hydrothermal pretreatment at 175ºC following either non-isothermal or partially isothermal heating regimes, was performed to improve the enzymatic saccharification of oyster mushroom SMS. Around 85-87% of the initial cellulose was recovered in the solid fraction after hydrothermal pretreatment. Hydrothermal pretreatment of oyster mushroom SMS at 175ºC improved enzymatic digestibility yielding solids with 60.0% and 55.7% cellulose and lignin content. Preparative enzymatic saccharification (PES) was performed using Cellic CTec2, validating the analytical results at a larger scale and showing effective cellulose and xylose saccharification from SMS compared to Cellic CTec3. In conclusion, SMS from shiitake and oyster mushrooms are promising sugar sources for microbial fermentations to end products of interest. The polysaccharides contained in shiitake SMS are readily hydrolysable, while those contained in oyster mushroom SMS require being pretreated to achieve a good saccharification.

1. Introduction

1.1. Mushrooms in today's world

Mushrooms are visible spore-bearing structures of fungi. Anatomically, they are fruitbodies, also known as fruiting bodies, that grow above the ground. Physiologically, they are sporocarps, which play a crucial role in the sexual reproduction of many fungi (Taylor and Ellison, 2010). Various mushrooms are classified as edible due to the lack of toxins and low levels of antinutrients. They have high nutritional value because they are rich in proteins, minerals, vitamins, and dietary fiber. Furthermore, their total fat content is low, but it is rich in unsaturated fatty acids as well as devoid of any cholesterol (Anmut et al., 2022). Regarding taste, mushrooms are considered delicious with a source of food compounds containing unique flavours. The phytochemical profile of mushrooms consists of various water-soluble substances such as organic acids, carbohydrates, nucleotides, free amino acids, polyols, as well as volatile carbon compounds (Gargano et al., 2017). The biochemical composition of mushrooms varies across species. Generally, the carbohydrate content of mushrooms ranges between 35 and 70% (w/w), that of proteins is 15–35%, whereas that of fats is below 5% (Kumla et al., 2020).

The production of meat has a major influence on the global climate because of the generated greenhouse gas emissions (Sunil at al., 2016). As an alternative, edible mushrooms offer a protein-rich source of food that can tackle climate issues provided that they replace meat at least partially (Paranagama et al., 2022). Some mushrooms, including edible ones, are referred to as 'medicinal mushrooms' because they contain an array of health-boosting components, such as peptides, proteins, β-glucans, and phenolic compounds, among others (Venturella et al., 2021). These compounds impart immunomodulatory, antibacterial, cytostatic, and antioxidant properties, which result in positive effects on the health of mushroom consumers. Consuming mushrooms has been associated with improved human health and wellness. Consequently, the marketing of mushrooms has become a global industry, whose production is increasingly demanded across all continents (Valverde et al., 2015). As a result, mushroom cultivation has grown more than thirty-fold since 1978 and is now a rapidly developing industry (Royse et al., 2017).

While most of the world's mushroom cultivation is centred in Asia, with China holding approximately 90% of the market share, mushroom cultivation has increased significantly in recent decades in the European Union (Martín et al., 2023b). Over fifty mushroom species are cultivated commercially, with the top-cultivated genera including *Pleurotus* (also known as

'oyster mushrooms') and *Lentinula* (mostly *L. edodes*, commonly referred as 'shiitake') (Shrikhandia et al., 2022). Most edible mushrooms cultivated worldwide are saprophytic fungi, also known as wood decomposers or wood-decay fungi. Saprophytic fungi are capable of breaking down lignocellulosic materials by producing an array of enzymes, including ligninases, cellulases, and hemicellulases. The products of wood degradation are utilized by the fungi as growth nutrients. As a result, mushroom cultivation is frequently linked with recycling significant volumes of agricultural and industrial waste (Kumla et al., 2020).

Owing to the expanding global population, the agricultural production has been continuously increasing during the last decades, and a doubling of today's levels is expected by 2050 (Choi and Labhsetwar, 2021). The expansion of the agriculture leads to an increase in the generation of crop residues, whose disposal poses a huge challenge. A way of dealing with crop residues, as well as forestry by-products, is to use them for formulating the substrate for the cultivation of mushrooms. That utilization of crop residues would avoid the need for environmentally harmful disposal methods like burning. Burning agriculture waste, which is a common disposing method, is an unsustainable practice, which releases harmful pollutants into the atmosphere and affects the soil health.

Cultivating mushrooms on agricultural residues represents a sustainable approach, where waste materials are recycled into valuable resources (Gupte et al., 2023). This sustainable approach not only addresses waste management but also provides a means of producing food sources. Moreover, mushroom cultivation offers a comprehensive approach to utilizing leftover lignocellulosic biomass. Unlike traditional agriculture, which is often affected by weather and seasonality, mushroom production can be carried out year-round indoors in a controlled atmosphere (Antunes et al., 2020).

Mushroom substrates are based on crop and forest residues, which are lignocellulosic materials, i.e., composed of lignin, cellulose, and hemicelluloses. However, they are supplemented with starch and nitrogen surces, such as cereal bran and legume flour, as well as mineral salts, to provide the required nutrients and structural support. During the cultivation phase, microbial and enzymatic activities degrade the substrate components, releasing nutrients that promote the growth of mushrooms and fungi. Fungal metabolism and the production of fruitbodies depend on the nutrients and energy sources released during the enzymatic breakdown of lignin, cellulose, and hemicelluloses present in the substrate (Kumla et al., 2020).

1.2. Spent mushroom substrate – problem and opportunities

The mushroom cultivation process concludes with the harvesting of the fungal fruitbodies, leaving behind a residual material known as spent mushroom substrate (SMS). SMS is an exhausted substrate that cannot be used for another growing cycle due to the depletion of nutrients. Around 3 – 5 kg of "spent" substrate is generated per kg of produced mushrooms (Zisopoulos et al., 2016). Due to the rapid growth of the mushroom market, the volume of generated SMS has been increasing steadily over time (Leong et al., 2022). The global output of SMS was estimated at 64 million tonnes in 2018, and it is expected to hit 100 million ton by 2026 (Mohd Zaini et al., 2023). While the fruitbodies are the primary food product, SMS is often perceived as an agricultural waste with little value.

Common disposal methods for SMS include spreading on land, burning in the open, incineration, composting with animal manure, and landfilling. However, these disposal methods are harmful to the environment and pose significant challenges to mushroom producers. These challenges include overloaded landfills and adverse environmental impacts of open burning or spreading on land. Additionally, due to the high moisture and low density of SMS, it is expensive to transport. On the other hand, drying fresh SMS is an energy-intensive process (Leong et al., 2022).

The improper disposal of SMS can also result in air and water pollution (Hu et al., 2021). The piles created during provisional storage of SMS can undergo spontaneous anaerobic digestion, resulting in the release of greenhouse gases (Beyer, 2011). Additionally, the degradation of organic matter in SMS may produce unpleasant odors that contribute to air pollution. Water bodies can become contaminated by leachate drainage from SMS piles, leading to pollution and eutrophication. The leachate may contain potentially hazardous compounds, posing risks to human health and aquatic ecosystems (Jiang et al., 2017).

The future growth of the mushroom-growing industry is threatened by the prevailing linear "take, make, dispose of" approach to SMS as waste. Transitioning to a circular economy model, where SMS is valued and efficiently utilized, is essential for fostering a sustainable mushroom business. If properly valorized, SMS can be considered a valuable by-product that holds significant industrial importance. SMS can be utilized in numerous ways, for example as a substrate for the production of biofuels (Martín et al., 2023b). The mushroom industry can contribute to sustainable development by addressing the environmental concerns linked with

SMS management and disposal. Efficient use of SMS not only enhances farm economy but also adds to the industry's long-term sustainability (Kosre et al., 2021). In the current thesis, SMS is used as a source of sugars that can be released by enzymatic saccharification of polysaccharides contained in SMS. Those sugars can then be used in microbial fermentations for producing biofuels, biopolymers, and platform chemicals.

1.3. SMS composition

Various factors, such as the type of mushroom species and the initial substrate, influence the composition of SMS. However, SMS primarily consists of a few fundamental constituents (Mohd Hanafi et al., 2018). This includes the main structural components of plant cell wall (lignin, cellulose, and hemicelluloses), which usually originate from the substrate materials, such as wood chips or agricultural residues, used in the cultivation. A part of the structural components is consumed during the cultivation, but the rest remains in the spent substrate. In addition to structural constituents, many other substances are also present in SMS. Some of them come from the mycelium, the thread-like structures that constitute the vegetative phase of fungi, which remains in the SMS after cultivation. The SMS also contains substances secreted by fungal growth, phytochemicals from the lignocellulose extractives, products of partial degradation of cell wall polymers of the substrate, as well as minerals derived from the substrate and fungal metabolism (Martín et al., 2023b).

1.4. Lignocellulosic biomass

Lignocellulosic biomass, also known as lignocellulose, is the cell-wall material from plant resources. Wood-processing by-products, such as sawdust and chips, forest and agricultural residues, energy crops, and food-processing wastes are major sources of lignocellulosic biomass. Lignocellulose is a sustainable source offering an array of potential uses, especially if it is processed by bioconversion in biorefineries for manufacturing biofuels, and other products of economic value (Martín, 2021).

Lignocellulose is composed of three main constituents: cellulose, hemicelluloses, and lignin. Cellulose and hemicelluloses are polysaccharides that make up to roughly 50 – 80% of the cell wall dry weight. These polysaccharides can be deconstructed into sugars by saccharification using acids or enzymes. The sugars resulting from saccharification processes can subsequently undergo fermentation to produce ethanol, other biofuels, and many other products. Lignocellulosic bioconversion into biofuels offers a viable and environmentally friendly

solution to meet the world's energy demands, reduce reliance on fossil fuels, and promote green and sustainable development (Zhang et al., 2021).

1.4.1. Cellulose

Roughly 40–60% weight of lignocellulosic biomass is composed of cellulose, making it an essential component. Cellulose is a glucan, i.e., a polysaccharide consisting of chains of repeated D-glucose units (Gandla et al., 2022). In cellulose macromolecule, glucose units are joined by β-1,4-glycosidic linkages (Fig. 1.1).

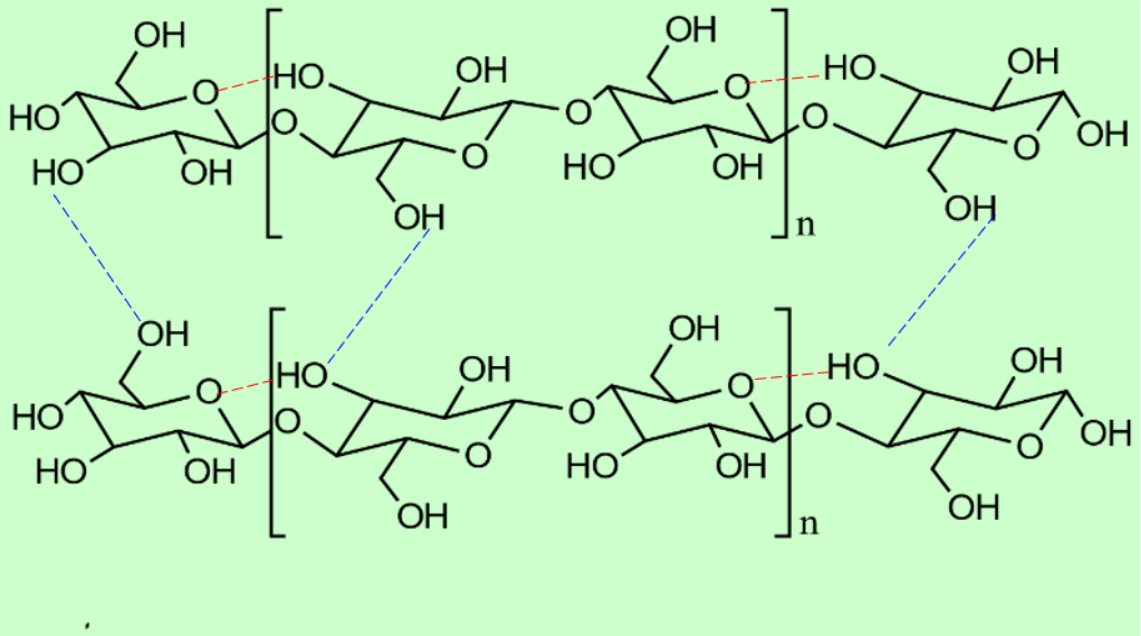

Figure 1.1. Cellulose's molecular framework. The red and blue dashed lines denote, respectively, intra- and intermolecular hydrogen bonds (Adapted from Gandla et al., 2022).

Cellulose chains form both crystalline and amorphous sections in the macromolecular structure (Martín et al., 2022). Cellulose macromolecules are devoid of any branching or coiling structures. Rather, they are linked with each other by hydrogen bonds. The hydrogen bonds are formed between the oxygen atom of a hydroxyl group on a glucose unit of one chain with the hydrogen atom of a hydroxyl group of either the same chain (intramolecular hydrogen bond) or a neighbouring one (intermolecular hydrogen bond). The hydrogen bonding gives cellulose a high tensile strength, making it an essential structural element of plant cell walls (Gandla et al., 2022).

The organization of cellulose fibrils within the lignocellulose matrix provides plants with the stability and support necessary to maintain their structural integrity. Natural cellulose exhibits a remarkably ordered structure, where the microfibrils are arranged not only via hydrogen

bonds, but also with contribution by Van der Waals forces. While the amorphous portions of cellulose are characterized by disrupted bonds and an irregular arrangement, the crystalline regions of the material are highly organized (Fig. 1.2). Cellulose molecules contain a reducing carbonyl group, at one terminus of the chain, while a non-reducing group is located at the other end.

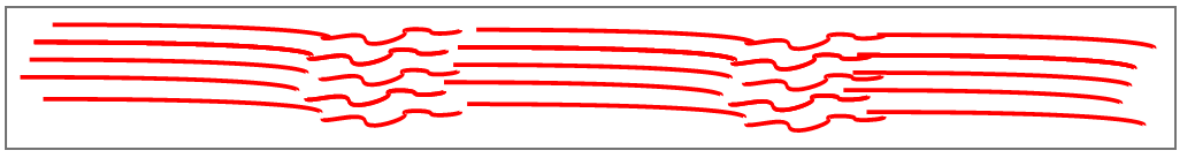

Figure 1.2. Representation of cellulose microfibrils. Straight lines depict crystalline regions, while wavy lines represent amorphous areas (Adapted from Gandla et al., 2022).

When cellulose undergoes incomplete hydrolysis, a disaccharide called cellobiose is the main product, while glucose is the product of the complete scarification. The full breakdown of cellulose into glucose is essential in enzymatic saccharification intended to generate sugars for subsequent bioconversion by microbial fermentation (French, 2017). In the study behind the current thesis, we aimed at a complete enzymatic saccharification to produce enough glucose to be used in fermentations with either oleaginous yeasts or biopolymer-producing bacteria. Those fermentations will produce microbial oil and exopolysaccharides within *MUSA* project, a research initiative linking mushroom production with agriculture sustainability (Martín et al., 2023a).

1.4.2. Hemicelluloses

Hemicelluloses make up 15–30% (w/w) of the total content of plant cell walls, ranking as the second polysaccharide in lignocellulose. Hemicelluloses, unlike cellulose, are heteropolysaccharides, i.e. heterogeneous polymers composed of several monosaccharides, mainly pentoses and hexoses, as well as uronic acid moieties and acetyl groups (Gandla et al., 2022). The pentoses most found in hemicelluloses are xylose and arabinose, while mannose, glucose, and galactose are the most common hexoses. The monosaccharide units in hemicelluloses are bonded together via glycosidic linkages.

The structure and composition of hemicelluloses may vary among various plant sources. For instance, hemicelluloses can make up as much as 40% of the dry weight of nonwood fibers, like grasses, but typically accounts for approximately 25–35% dry weight of wood fibers. Furthermore, the structure and composition of hemicelluloses differ in softwoods and

hardwoods, reflecting the diversity of plant species and tissues (Rajinipriya et al., 2018). Xylans are the most prevalent hemicelluloses in the cell walls of hardwoods and agricultural residues (Gandla et al., 2022). Xylans are polysaccharides consisting of xylose units linked via β-1, 4-glycosidic bonds. They often contain glucuronic acid and/or α-L-arabinofuranose in the side chains. The xylan content in hardwoods can vary from 10 to 35% (w/w), while softwoods typically have a xylan content ranging from 10 to 15% (w/w). Based on their composition, xylans can be categorized into three main groups: glucuronoxylan, glucuronoarabinoxylan, and arabinoxylan. Softwood xylans are mainly made up of arabino-4-O-methylglucuronoxylans, whereas O-acetyl-4-O-methylglucuronoxylan is the primary component of hardwood xylans (Fig. 1.3).

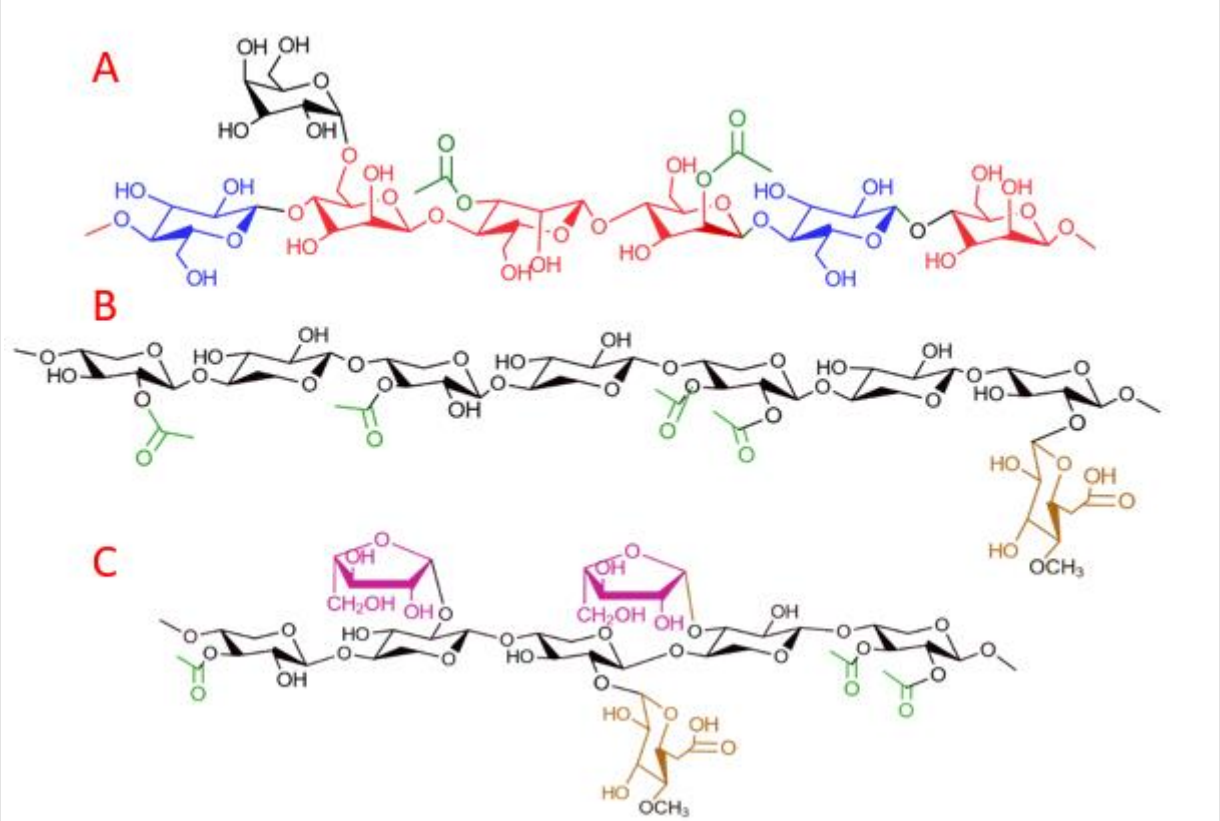

Figure 1.3. Schematic representation of hemicelluloses. (A) Softwood O-acetyl-galactoglucomannan, featuring hexapyranosic structures in blue (glucose), red (mannose), and black (galactose). Green represents acetyl groups. (B) Hardwood O-acetyl-4-O-methylglucurono-D-xylan, showcasing xylose (black) and glucuronic acid (brown) units. Acetyl groups are depicted in green and methyl groups in black. (C) Gramineous arabino-(O-acetyl-4-O-methylglucurono)-D-xylan. Xylose, glucuronic acid, arabinose, and acetyl groups

are represented in black, brown, pink, and green, respectively. (Adapted from Gandla et al., 2022).

Hardwood xylans are heavily acetylated, which is positive for their water solubility (Córdova et al., 2019). Therefore, many different enzymes are required for their hydrolysis.

Hemicelluloses efficiently seal the spaces between microfibrils in the cell wall by forming hydrogen bonds with cellulose fibrils. The carboxyl group of glucuronic acid, a hemicellulosic constituent, promotes fiber delamination within the cell wall structure. Due to its more open structure, which enables it to draw in and bind more water molecules, hemicelluloses are more hygroscopic than cellulose. Wood hemicelluloses have a degree of polymerization, i.e. a number of monosaccharide units in a macromolecule, of up to 200, which is considerably lower than that of cellulose (Gandla et al., 2022).

1.4.3. Lignin

Lignin is the third main component of plant cell wall. It is a complex organic polymer that makes up to about 20 to 35 percent of the dry mass of wood. Lignin is essential for cell wall development and provides plant tissues with structural rigidity and strength. Lignin is a heterogeneous aromatic biopolymer with an amorphous nature. It can be regarded as a polyphenol made of phenylpropanoid units, which are arranged in a three-dimensional configuration (Mahmood et al., 2018). The building blocks of lignin are three phenylpropanoid derivatives known as guaiacyl, syringyl, *p*-hydroxyphenyl units, which are linked with different bonds including carbon-carbon or ether linkages (Fig. 1.4).

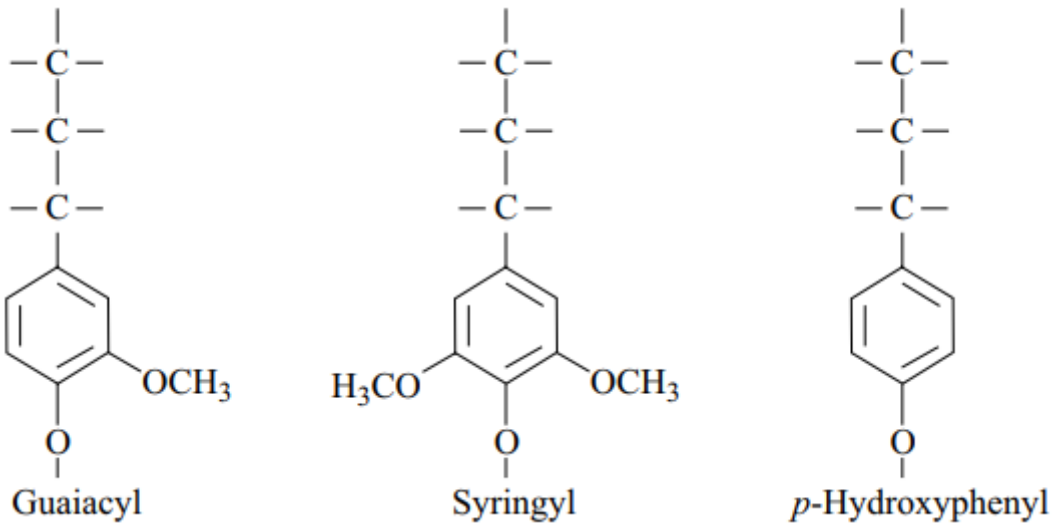

Figure 1.4. Main phenylpropanoid units in lignin (Adapted from Gandla et al., 2022).

The phenylpropanoid units derive from three precursors, namely coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol (Gandla et al., 2022). The synthesis of the lignin macromolecules is produced from the three precursors through polymerization processes. As a result, a cross-linked and dense network is formed inside the cell wall matrix. Isolated lignin usually has a molecular weight of 1000–20 000 g/mol. Lignin strengthens cell wall formation by bridging gaps between cellulose, hemicelluloses, and pectin components. Lignin biosynthesis takes place in the cytosol of plant cells, where several enzymes and metabolic processes participate in the polymerization of lignin precursors (Ekeberg et al., 2006).

Lignin content is typically higher in woody materials than in agricultural residues. Furthermore, lignin composition varies with the plant species. In softwood, lignin is composed of guaiacyl units, while in hardwood, both guaiacyl and syringyl units are important constituents. On the other hand, in gramineous plants, the three phenylpropanoid units are contained in substantial proportions (Gandla et al., 2022).

Lignin is relevant for this study because of its effect on cellulose saccharification. The reactivity of cellulose during enzymatic saccharification or chemical processing is affected by the close association of lignin in the so-called lignin-carbohydrate complexes (Gandla et al., 2022). Therefore, for having an effective enzymatic saccharification of cellulose it is advantageous to remove lignin, which can be achieved by running a pretreatment process.

1.5. Pretreatment for enzymatic saccharification

Pretreatment is an important step that is performed prior to the enzymatic saccharification in bioconversion of lignocellulosic biomass (Fig. 1.5) Pretreatment is directed to increase the accessibility of cellulose to saccharifying enzymes, thereby enhancing digestibility and increasing product yields. Pretreatment deconstructs the intricate structure of lignocellulosic biomass making it more prone to enzymatic saccharification (Jönsson and Martín, 2016).

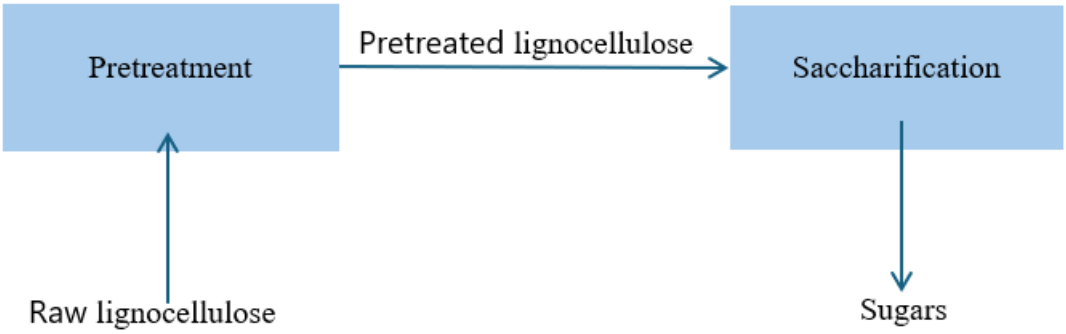

Figure 1.5. Schematic representation of the pretreatment and enzymatic saccharification in bioconversion of lignocellulosic biomass.

An effective pretreatment should result in a high enzymatic digestibility of cellulose with a minimal formation of inhibitory by-products (Jönsson and Martín, 2016). Some of the mechanisms behind the enhancement of enzymatic digestibility by pretreatment are removal of hemicelluloses or lignin, disrupting the lignin-carbohydrate complexes, decreasing the crystallinity of cellulose, or increasing the porosity of the substrates (Zhao et al., 2012).

1.5.1. Hydrothermal pretreatment

Many different pretreatment techniques have been developed and several other methods are under research and development. All of them are aimed at reducing or eliminating barriers contributing to biomass recalcitrance (Galbe & Wallberg, 2019). Most pretreatment techniques operate at high temperatures. High temperatures are particularly effective in disrupting biomass structure and improving enzymatic saccharification.

Most of the pretreatment methods tested in the industry are hydrothermal processes. In hydrothermal pretreatment (HTP), lignocellulosic biomass is mixed with an aqueous medium, and then heated for certain time (Martín et al., 2022). HTP is a well-established and effective technique that has been successfully used with a range of feedstocks and is regarded as a technologically advanced method. Liquid hot water (LHW) pretreatment is one of the fundamental methods of hydrothermal pretreatment. During LHW pretreatment, the raw material is suspended in water and heated to around 200°C under high pressure for a predetermined period. This procedure involves several significant alterations, such as slight removal and relocalization of lignin, partial solubilization of hemicelluloses, and hydration of cellulose fibers. Furthermore, HTP also enlarges the internal pore sizes of biomass fibers, which enhances the surface area accessible for the enzyme infiltration, thus improving the efficiency of the cellulose saccharification.

Hydrothermal pretreatment can be auto-catalyzed, or catalyzed with chemicals, e.g., sulfuric acid or sulfur dioxide. Commercial-scale hydrothermal pretreatment has proven effective in producing a range of products, such as molasses, lignin pellets, and ethanol (Gandla et al., 2018). HTP is relevant for this thesis because it was used for enhancing cellulose saccharification in the SMS that displayed a poor enzymatic digestibility.

1.5.2. Biological pretreatment

Hydrothermal pretreatment and other conventional methods have the drawback of requiring heating to a high temperature, which is costly. Biological pretreatment with lignin-degrading fungi is a low-cost alternative. White-rot fungi have the capability to efficiently decompose lignin, which is the most resistant structural element of plant cell walls. White-rot fungi produce enzymes that break down lignin into CO_2, thereby contributing to the carbon cycle and facilitating the decomposition of organic materials in forest ecosystems. By harnessing the abilities of lignin-degrading fungi, lignin content in lignocellulosic biomass can be decreased rendering a biomass material with a higher susceptibility of cellulose to enzymes. That allows implementing a low-temperature pretreatment for enhancing the enzymatic saccharification of cellulose (Wan and Li, 2012). Using biological pretreatment, the cost are reduced in comparison to conventional methods requiring high temperatures.

A drawback of biological pretreatment is its slow rate and the partial consumption of carbohydrates by many fungi. Therefore, biological pretreatment as a stand-alone method has a limited viability (Chen et al., 2022). Using cultivation of edible fungi as an approach of biological pretreatment opens new bioconversion possibilities. It allows the combined production of food (mushroom fruitbodies) and fermentable sugars (to be converted to biofuels, biopolymers, or platform chemicals), which improves the cost-effectiveness of the pretreatment (Fig. 1.6)

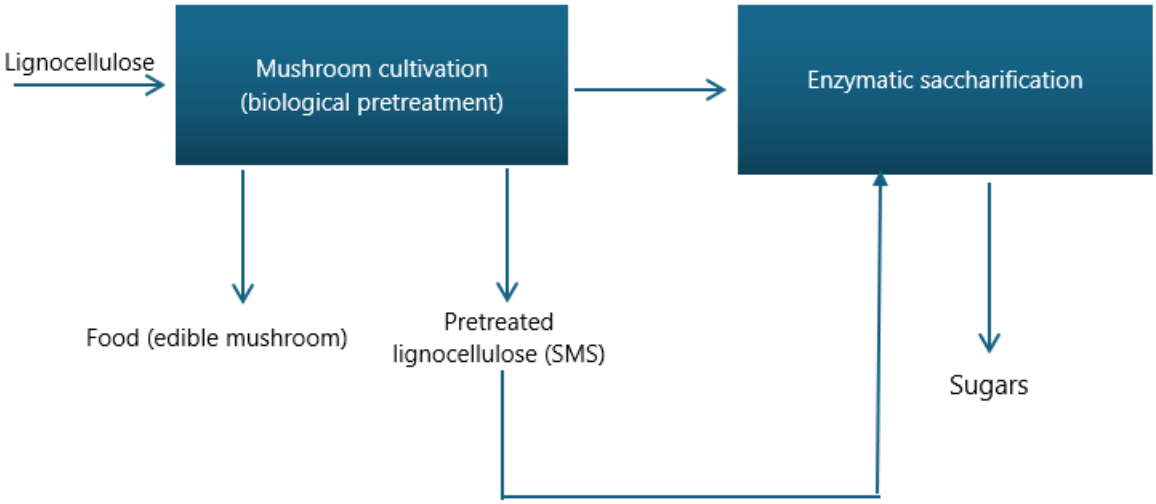

Figure 1.6. Schematic representation of the bioconversion process using mushroom cultivation as pretreatment prior to enzymatic saccharification.

Xiong et al. (2018) demonstrated that the SMS resulting from the production of shiitake mushroom (*Lentinula edodes*) has high susceptibility to enzymatic saccharification, and the resulting hydrolysates were shown to be readily fermentable by *Saccharomyces cerevisiae* (Chen et al., 2022). The enzymatic saccharification of SMS of oyster mushrooms (*Pleurotus* spp.) has also been investigated. However, the literature lacks reports on comparison of the enzymatic saccharification of SMS of shiitake and oyster mushrooms. This thesis provides the first systematic evaluation of the enzymatic saccharification of shiitake and oyster mushrooms.

1.6. Enzymatic saccharification of lignocellulose biomass

The enzymatic saccharification is a key stage in bioconversion of lignocellulosic biomass through the sugar-platform route (Gandla et al., 2022). In the sugar-platform bioconversion, sugars are produced by saccharification of lignocellulose polysaccharides, and then converted to end products, such as ethanol, butanol, lactic acid, etc., by fermentation with different microorganisms (Fig. 1.7).

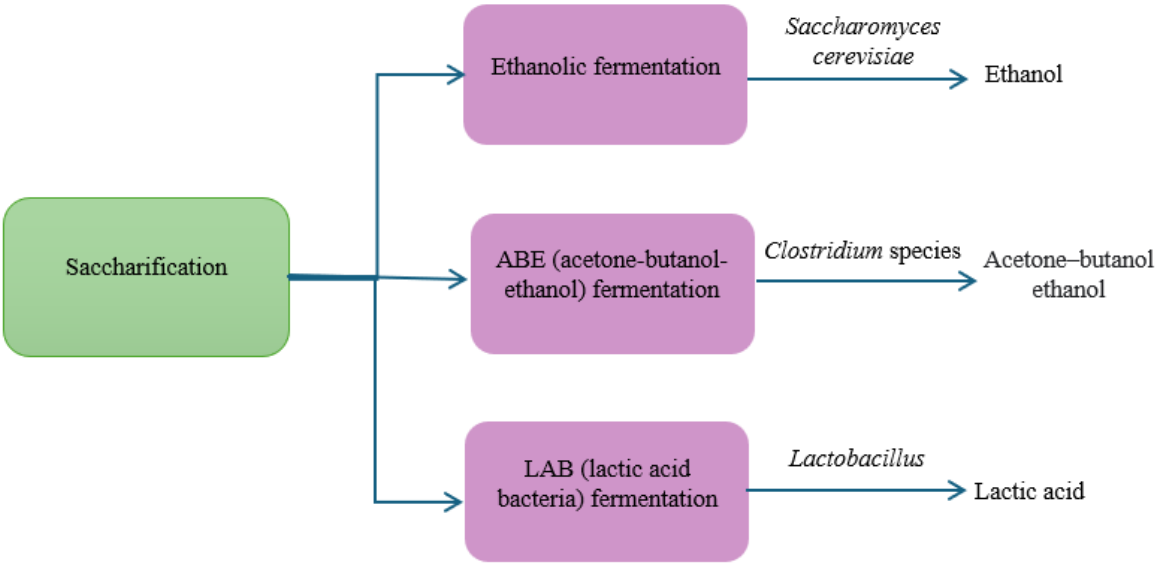

Figure 1.7. Alternatives of bioconversion pathways by microbial fermentations of sugars produced by biomass saccharification.

Considerable emphasis has been placed on research projects aimed at enhancing the efficiency of enzymatic saccharification for economically viable bioethanol production. The utilization of the enzyme complex of the fungus *Trichoderma reesei* has been a main research focus for developing enzymatic preparations able to effectively hydrolyse cellulose. The glycoside hydrolases, i.e. enzymes that catalyse the hydrolysis of glycosidic bonds, of *T. reesei* enzyme complex are very effective for saccharification of cellulose and hemicelluloses. Enzymes obtained from *T. reesei* often form the basis of commercial enzyme cocktails used in industrial settings (Gandla et al., 2022). In the enzyme cocktails, complementary and synergistic activities of various enzymes, such as cellulases, hemicellulases, and pectinases, contribute to an effective saccharification. These cocktails are employed in a variety of biorefinery processes, such as the production of cellulosic ethanol, and are designed to achieve high levels of cellulose hydrolysis.

In addition to *T. reesei*, many other fungi and some bacteria produce glycoside hydrolases, but not all of them produce the enzyme activity titers required for commercial application. The cellulolytic enzyme-producing capabilities of fungi, such as *Aspergillus* spp. and *Penicillium* spp., are of high interest (Passos et al., 2018). Studies have shown that combining enzymes from *T. reesei* with enzymatic extracts from these fungi to create cocktails can provide excellent alternatives for effectively hydrolyzing plant biomass (Pimentel et al., 2021).

There has been a global focus on research aimed at identifying novel microbes capable of producing cellulolytic enzymes with higher specificity and increased efficiency (Hyeon & Han, 2022). In addition to fungi and bacteria from terrestrial ecosystems, recent research has identified marine microorganisms that are particularly attractive sources of cellulolytic enzymes due to their diverse habitats (Kennedy et al., 2011).

1.6.1. Glycoside hydrolases

Glycoside hydrolases include different kinds of cellulases and hemicellulases, i.e, enzymes that degrade cellulose and hemicelluloses, respectively. Cellulases are responsible of the saccharification process by breaking down the glycosidic bonds present in cellulose in an intricate reaction catalyzed by multiple enzymes. Fungal cellulases include several enzyme groups, such as endoglucanases, cellobiohydrolases, and β-glucosidases. The modular structure of cellulases consists of domains or modules, which are units that fold independently of one another. A short poly-linker segment bridges the catalytic domain, typically located at the N-terminal region of a cellulase, with the carbohydrate-binding domain (CBD) found at the C-terminal end. In general, glycoside hydrolases exhibit optimal activity within the temperature range of 40 to 80°C and pH levels ranging from 4 to 6.5. However, the precise ideal circumstances could change based on the enzyme's properties and source (Wyman et al., 2004).

The enzymatic hydrolysis of cellulose starts by the action of endoglucanases, which breaks down the β-1,4-glycosidic bonds in the macromolecule. Endoglucanases have traditionaly been categorized as endo-acting cellulases because they were assumed to hydrolyze β-1,4-glycosidic linkages within the cellulose chain. Their activity can be measured using soluble cellulose substrates, such as in the carboxymethylcellulase test (CMCase). Endoglucanases are usually active on the amorphous portions of cellulose. Recent data suggest that some cellulases exhibit both endo- and exo-acting mechanisms (Himmel et al., 2018). As a result of endoglucanase action, the long cellulose chain is split into fragments of shorter chain length including cello-dextrins and cello-oligosaccharides. After that, exocellulases, also referred to as cellobiohydrolases, hydrolyse glycosidic bonds close to the end of the chains, which results in formation of the disaccharide cellobiose. Exoglucanases hydrolyze β-1,4-glycosidic linkages from the ends of chains (Estela & Luis, 2013). Endoglucanases and exoglucanases exhibit a high degree of synergy, which is necessary for the effective degradation of cellulose crystals (Maki et al., 2009). Finally, β-glucosidase, also known as cellobiase, hydrolyses the β-1,4-glycosidic bond of cellobiose with formation of glucose (Fig. 1.8.)

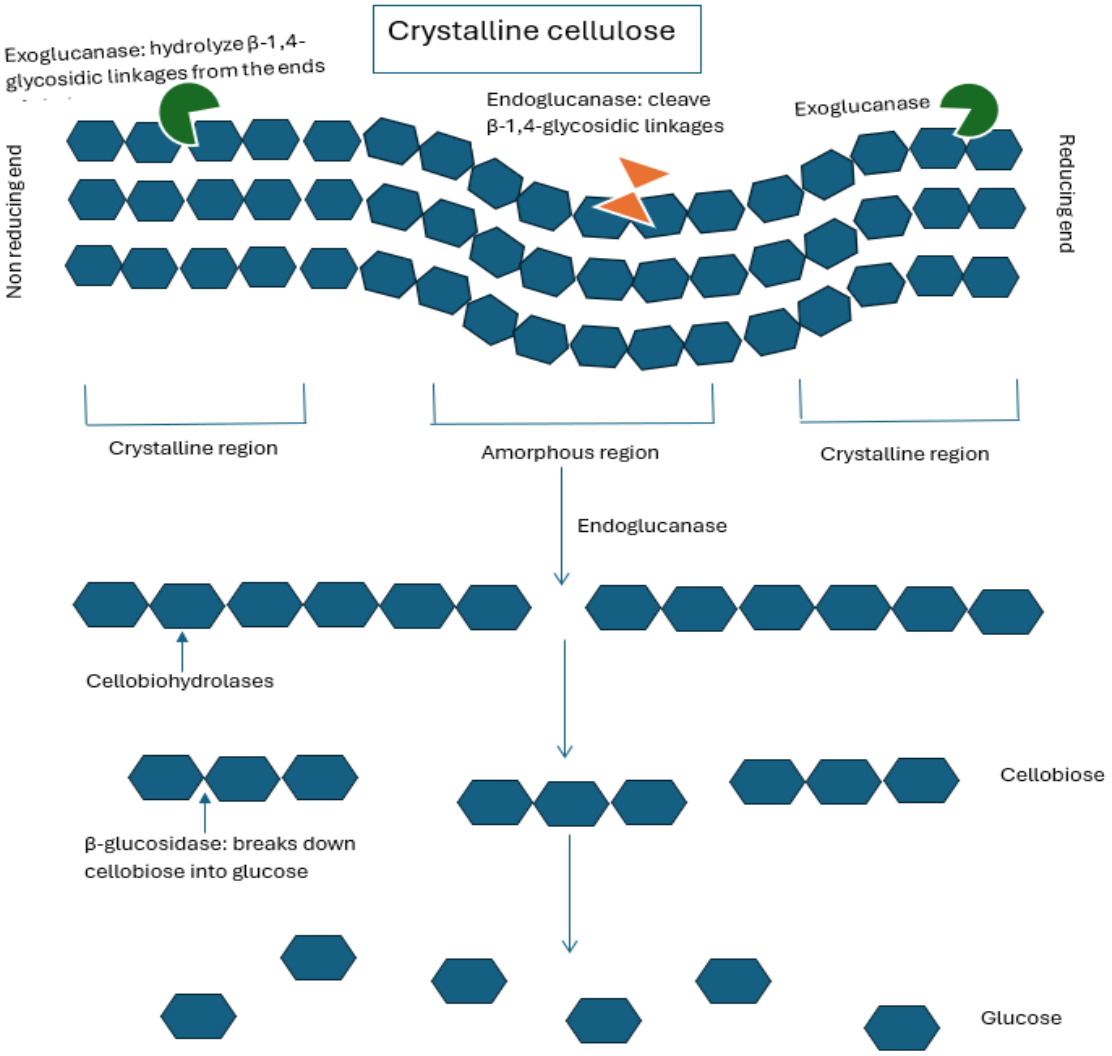

Figure 1.8. Schematic representation of cellulose hydrolysis.

1.6.2. Other hydrolytical enzymes

In addition to glucoside hydrolases, several other enzymes are required to complete hydrolysis of lignocellulose polysaccharides (Gandla et al., 2022). For example, polysaccharide lyases catalyse the splitting of the uronic acid moieties of hemicelluloses. Furthermore, carbohydrate esterases catalise the splitting of ester bonds in hemicelluloses. Examples of carbohydrate esterases are of acetyl esterases and feruloyl esterases, which calalyse the removal of acetyl groups and ferulic acid moieties.

1.6.3. Auxiliary activities

Some other enzymes contribute to deconstruction of cellulose following a non-hydrolytical mechanism. That includes lytic polysaccharide monooxygenases (LPMOs) and cellobiose dehydrogenase, which are oxidoreductases (Gandla et al., 2022). LPMOs were discovered recently (Horn et al., 2012) and they have become very useful for the saccharification of

recalcitrant lignocellulosic materials. LPMOs break glycosidic bonds of cellulose using an oxidative mechanism, rendering cellulose chains shorter fragments, which facilitates the hydrolytic action of the cellulases. LPMO requires the presence of molecular oxygen and an electron donor for their action. Lately, some producers of enzymes commercialize improved cellulolytic cocktails containing added LPMOs (Müller *et al.*, 2015).

1.7. Aim of the study

Several studies have demonstrated the potential of cultivation of shiitake mushroom (*L. edodes*) as a biological pretreatment for enhancing the enzymatic saccharification of cellulose. The cultivation of oyster mushrooms (*Pleurotus* spp.) has shown some potential, but the results are rather dispersed and not conclusive.

The aim of the current study was to perform a thorough investigation of the enzymatic saccharification of spent substrates from cultivation of *L. edodes* and *P. ostreatus* to gather experimental data for a comparative assessment of the effectiveness of both fungal species as biological pretreatment agents for lignocellulosic biomass.

2. Materials and Methods

2.1. Materials

2.1.1. Spent mushroom substrate (SMS)

SMS samples from the cultivation of two different fungal species, namely oyster mushroom and shiitake (*Lentinula edodes*), were used. The oyster mushroom SMS corresponded to a *Pleurotus ostreatus* × *Pleurotus eryngii* hybrid strain, hereafter referred to as Black Pearl King Oyster (BPKO) mushroom. BPKO SMS was provided by Husbonden AS (Disenå, Norway). The SMS was collected at the end of the cultivation of a BPKO strain grown on an initial substrate containing 60% (w/w) lignocellulose and 40% water. The initial substrate also contained 0.08% (w/w) of $CaCO_3$.

Two types of oyster mushroom SMS, namely one based on a conventional initial substrate and another one based on non-conventional initial substrates, were used. The lignocellulosic part of the conventional substrate consisted of oak sawdust and wheat bran, which represented, respectively, 80 and 20% (w/w) of the dry weight. Four SMS based on different non-conventional initial substrates using biomass residues from Innlandet were included in the study. The lignocellulosic components of the four non-conventional substrates consisted of (i) wheat straw, (ii) spelt straw, (iii) oat straw, and (iv) and oak sawdust and coffee chaff.

The shiitake SMS was provided by the Swedish University of Agricultural Sciences. It was collected after shiitake cultivation on an initial substrate based on birch sawdust (80%) and wheat bran (20%).

2.1.2. Enzymes

Three enzyme preparations, namely two commercial mixtures and one experimental cocktail, were used in the analytical enzymatic saccharification. The commercial preparations were the cocktail Cellic CTec2 commercialized as *Cellulase, enzyme blend* by Sigma-Aldrich (Steinheim, Germany) and a mixture of cellulases produced by *Trichoderma reesei* and commercialized as *Trichoderma* cellulases (Sigma-Aldrich). Cellic CTec2 consists of β-glucosidase, cellulases, other cellulose-degrading enzymes, and hemicellulases. The experimental cocktail (ExpC) is a raw mixture from a fermentation broth, and it was supplied by a major enzyme producer under MTA conditions. Additionally, a state-of-the-art preparation, Cellic CTec3 HS (Novozymes, Bagsværd, Denmark), was used as reference in the

preparative enzymatic saccharification study. The optimal temperature range of Cellic Ctec2 and *Trichoderma* cellulases is 45-50°C.

2.1.3. Chemicals

All the chemicals used in this study were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and Merck (Darmstadt, Germany).

2.2. Characterization of the SMS

The used SMS were characterized by compositional analysis. Standard methods were used for determination of the content of dry matter, extractive compounds, mineral components, structural polysaccharides, and lignin. All the analyses were conducted in triplicates, and the mean values and standard deviations were calculated. Before starting the analysis, the SMS samples were subjected to drying and shredding for making them ready for the experiments.

2.2.1. Preparation of the SMS for the experiments

The SMS samples were received wet from the suppliers. Drying was performed at room temperature until a dry-matter (DM) content above 90% (w/w) was reached. The particle size of the dry samples was reduced by using a Robot Coupe shredder (Robot-Coupe SNC, Montceau-en-Bourgogne, France). The shredded material was then, and the fraction with particle-size below 0.5 mm was collected and stored in sealable plastic bags until further utilization.

2.2.2. Dry matter determination

The dry matter content of the SMS was determined gravimetrically after drying an aliquot of each material at 105°C overnight. A methodology described previously by Martín et al. (2006) was followed. The samples were weighed using an analytical balance (Sartorius Lab Instruments GmbH, Göttingen, Germany) before and after drying. The drying was performed by heating the samples in a Termaks TS4057 oven (Termaks, Bergen, Norway). The dry matter of samples generated during processing the SMS was determined using a moisture analyzer (Mettler-Toledo GmbH, China).

2.2.3. Ash determination

The content of mineral components was determined as ash after incinerating two grams (dry weight (DW**)**) samples in tared porcelain crucibles at 575°C for five hours in a Carbolite CWF 1100 muffle furnace (Carbolite Gero, Sheffield, UK) following the National Renewable Energy Laboratory (NREL) standard protocol on ash determination (Sluiter et al., 2005a). Before the

analysis, the crucibles were prepared by heating them in the muffle furnace under the same conditions used in the sample incineration. By the end of the incineration of the samples, the crucibles with the ash were taken out of the furnace and transferred into a desiccator. Following this, the crucibles were weighed, and the ash content was calculated as follows.

$$Ash\% = \frac{Weight_{crucible\ plus\ ash} - Weight_{crucibe}}{ODW_{sample}} x100$$

2.2.4. Determination of extractive compounds

The content of extractive compounds was quantified after sequential extraction using distilled water and 96% ethanol as solvents. An NREL standard method (Sluiter et al., 2005b) was used. A 10-g aliquot of biomass was extracted in a Soxhlet apparatus with 250 mL of each of the solvents, maintaining a solvent-to-biomass ratio (SBR) of 25:1 (v/w). The SMS sample was placed in a Cytiva's Whatman cotton cellulose thimble (Alundum thimbles, China), which was then inserted into the extraction chamber of the Soxhlet apparatus. Both extractions were conducted for eight hours. In the water extraction, a minimum of 35 min was required for each siphon cycle, while in the ethanol extraction, each cycle lasted around 15 min. When the extraction process was completed, the extracts were preserved at 5ºC in the refrigerator, except a 10-mL sample that was withdrawn and kept frozen for subsequent analyses. The extract-free solids were air dried until a constant weight was achieved. For determining the extractive yield, 1-mL aliquots of the extracts were dried overnight at 105ºC (Termaks), and the extract was weighed using an analytical balance (Sartorius). The extractives' yield (EY) was calculated as the mass percentage of extract out of the mass of the SMS sample submitted to extraction.

2.2.5. Determination of structural carbohydrates and lignin

Analytical acid hydrolysis (AAH) was used to determine structural carbohydrates and lignin. A standard protocol developed by NREL researchers was followed (Sluiter et al., 2008). In glass tubes, 300 mg (DM) samples were placed, and 3 mL of a 72% (w/w) sulfuric acid solution was added. Several samples were processed in parallel, and the sulfuric acid addition was performed sequentially with one-minute intervals between samples, followed by a one-hour incubation in a water bath at 30°C. After that, the suspensions were transferred into pre-weighed 100-mL blue-cap flasks and diluted with extra water to achieve a sulfuric acid concentration of 4% (w/w). The reaction mixtures were subjected to dilute-acid hydrolysis at 121°C for one hour using a CV-EL autoclave (CertoClav Sterilizer GmbH, Leonding, Austria). At the end of the hydrolysis, the hydrolysates were separated from the solid residue (Klason lignin) via filtering

through pre-weighed glass filters. After drying overnight at 105ºC in a Termaks oven, gravimetric analysis was used to determine Klason lignin as the solid residue. For subsequent analysis, 1-mL hydrolysate samples were stored frozen in Eppendorf tubes. The sugars in the analytical acid hydrolysates were determined by high-performance liquid chromatography (HPLC). The content of various polysaccharides (glucan, xylan, arabinan) was determined based on the concentration of the monosaccharides (glucose, xylose, arabinose) in the hydrolysates. The relation between the molecular masses of anhydrosugars and sugars was included in the calculation as a correction for the water addition during hydrolysis.

2.2.6. Quantification of sugars in hydrolysates

Sugar concentrations in all hydrolysates were quantified by HPLC. For the quantification of glucose, xylose, and arabinose in the analytical acid hydrolysates, an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) was used. The column was maintained at 60ºC for ensuring an efficient sugar separation. Samples were diluted and filtered with a 0.45 µm filter, and a volume of 10 µL was injected to the HPLC system. The eluent was a 0.05 M aqueous solution of sulfuric acid, and it was supplied at a flow rate of 0.6 mL/min. A refractive index (RI) detector was used for the detection of the sugars. The peak areas were integrated manually to distinguish and detect prominent peaks and shoulders.

In the enzymatic hydrolysates, glucose and xylose were separated with Rezex-RPM Monosaccharide Pb^{2+} column (Phenomenex, Torrance, CA, USA) heated to 85ºC and an RI detector (Showa Denko, Tokyo, Japan). The eluent was distilled water, filtered through a 0.45 µm membrane filter of regenerated cellulose (GE Healthcare, Buckinghamshire, United Kingdom), and eluted at 0.6 mL/min for 40 min per sample. Before HPLC analysis, all the samples were appropriately diluted and filtered using 0.45 µm Nylon syringe filters (VWR, Radnor, PA, USA).

2.3. Hydrothermal pretreatment

Hydrothermal pretreatment of oyster mushroom SMS was performed by heating a mixture containing 30 g (DW) of dry SMS suspended in 268 mL of distilled water in a pressurized Parr 4520 reactor (Parr Instrument Company, Moline, IL, USA). Two regimes were applied. In one regime, the SMS suspension was treated non-isothermally by heating it to 175 °C and cooling it to room temperature immediately afterward. In the second regime, the temperature was held at 175 °C for 30 min before cooling to room temperature by passing cold water through an internal coil controlled by a solenoid valve module.

2.4. Determination of the activity of the enzyme preparations

Various activities of the used enzyme preparations were determined before saccharification. That allowed to use similar dosages for all the preparations in the saccharification assays.

2.4.1. Filter paper activity (FPA)

Total cellulase activity was determined as FPA according to the procedure developed by Ghose (Ghose, 1987). A Whatman No. 1 filter paper strip (1 × 6 cm) weighing approximately 50 mg was used as substrate. Aliquots of 0.5 mL of diluted cellulase were added to the filter paper strips in test tubes. The tubes were incubated at 50 °C in a water bath for exactly 60 min. At the end of the incubation, the tubes were removed from the bath, and the cellulase reaction was stopped immediately by adding 3.0 mL of 3,5-dinitrosalicylic acid (DNS) reagent. All tubes were boiled for exactly 5.0 min in a vigorously boiling water bath. Finally, the coloured solution was diluted with 20 mL of deionized water, and the absorbance was measured at 540 nm in a spectrophotometer (UV 3100P spectrophotometer (VWR, Leuven, Belgium).

2.4.2. Carboxymethylcellulase (CMCase) activity

CMCase was used as a measure of the endoglucanase activity of the enzyme preparations. A cellulose derivative, 2% carboxymethylcellulose (CMC), was used as substrate, and the DNS reagent was used for quantification of the released reducing sugars. A 2% CMC solution was used, and the Ghose's protocol was adopted (Ghose, 1987) using different enzyme dilutions (1:100, 1:200, 1:500, 1:1000, and 1:2000). The assay was performed at pH 4.8 and 50 °C for 30 min. After elapsing the incubation time, 3 mL of DNS solution was added to stop the reaction and the test tubes with the reaction mixtures were placed in a boiling water bath for 30 min. The absorbance was read at 540 nm using spectrophotometer. One unit (U) of enzyme activity was defined as the quantity of enzyme, which released 1 μmoL/minute of glucose under the standard assay conditions.

2.4.3. Xylanase activity

The DNS colorimetric procedure with xylose as standard was used to measure the xylanase activity (Miller 1959). The assay mixture was rather the same as in the cellulase activity assays, but beechwood xylan was used as substrate. The mixture consisted of 0.5 mL of a 2% beechwood xylan solution in sodium citrate buffer at pH 4.8, along with 0.5 mL of the enzymatic solution at standard dilutions. Under assay conditions, one unit of enzyme activity corresponded to the amount of enzyme capable of liberating 1 mol of xylose per minute.

2.5. Enzymatic saccharification

2.5.1. Analytical enzymatic saccharification (AES)

Analytical enzymatic saccharification was performed at either 5 or 10-% (w/w) solids load. Around 50 mg (DM) of SMS suspended in 900 μL of 50 mM sodium citrate buffer (pH 5.2) was used for the experimental runs performed at 5% solids. For the trials at 10-% (w/w) solids content, 100 mg (DM) were used, and the volume of the buffer solution was 850 mL. The reaction suspensions were prepared in 1.5-mL Eppendorf tubes. The tubes with the suspensions were mixed by vortexing at room temperature and placed in a Termaks B4115 incubator for one hour set at 45 ºC. After that, 50 μL of a previously prepared stock solution containing the exact amount of enzyme for achieving the dosage required in each experiment was added. After that, the saccharification was run for 72 h. By the end of the hydrolysis, the tubes were centrifuged at 20,000x g for 5 min. The supernatant was recovered, and the solid residue was discarded. A portion of the supernatant was used for HPLC analysis after an appropriate dilution and the rest was stored frozen. The glucose values quantified by HPLC were used for calculating the enzymatic digestibility of cellulose and the glucose yield. The enzymatic digestibility was determined as the mass percentage of the initial cellulose in the assay that was saccharified into glucose. All the experiments were run in triplicates.

2.5.2. Evaluation of the SMS saccharification using different enzyme preparations

The AES assay was used for assessing the effectiveness of different enzyme preparations in the saccharification of SMS. The experiment was performed with the SMS from shiitake and oyster mushroom using the enzyme preparations Cellic CTec2, *Trichoderma* cellulases, and ExpC. Two different enzyme dosages and two substrate loadings were used as shown in (Table 2.1).

Table 2.1. Experimental conditions used for the evaluation of the enzymatic saccharification of SMS of shiitake and oyster mushrooms.

Enzyme preparation	Enzyme dosage, FPU/g	Substrate loading, % (w/v)
Cellic CTec2	25	5
Cellic CTec2	25	10
Cellic CTec2	15	5
Cellic CTec2	15	10
Trichoderma cellulases	25	5
Trichoderma cellulases	25	10
Trichoderma cellulases	15	5
Trichoderma cellulases	15	10
ExpC	25	5
ExpC	25	10
ExpC	15	5
ExpC	15	10

2.5.3. Enzymatic saccharification of extract-free SMS

The enzymatic conversion of the extract-free SMS, i.e. the solid residues after removal of the extractives, was evaluated. The experiment was performed with samples of the extract-free SMS of shiitake and oyster mushrooms. Trials with the raw SMS were performed as references for comparison. The trials were run with two enzyme dosages and two substrate loadings using the three enzyme preparations mentioned in the previous subsection.

2.5.4. Enzymatic saccharification of SMS from non-conventional initial substrates

The enzymatic saccharification of SMS resulting from oyster mushroom cultivation on four non-conventional substrates (based on straws of wheat, oat, and spelt, and oak sawdust combined with coffee chaff) was evaluated. As reference, the experiment included SMS from cultivation of shiitake and oyster mushrooms on conventional substrates (based on sawdust of birch and oak, and wheat bran). The saccharification was conducted with the enzyme preparation Cellic CTec2 using 25 FPU/g as the enzyme dosage and 5% (w/v) as the substrate loading.

2.5.5. Enzymatic saccharification of hydrothermally pretreated SMS

Hydrothermal pretreatment was applied to oyster mushroom SMS. The solid material resulting from the pretreatment was subsequently submitted to enzymatic saccharification. The enzyme preparation Cellic CTec2 at a dosage of 25 FPU/g and 15 FPU/g was used as the enzyme dosage. The substrate loading was 5% and 10% (w/v).

2.5.6. Preparative enzymatic saccharification

An additional experiment was performed to validate at a larger scale the experimental conditions resulting in the best results in the analytical enzymatic saccharification study. Since the experiment also aimed at producing hydrolysates for microbial fermentations, it is hereafter referred to as preparative enzymatic saccharification. The experiment was performed using shiitake SMS as substrate at a 10-% (w/w) solids load. The enzyme preparations Cellic CTec2 and Cellic CTec3 HS were used. The experiment was run in duplicates.

For each experimental run, around 60 g (DM) of SMS was suspended in 510 mL of 50 mM sodium citrate buffer (pH 5.2) in a 2-L Erlenmeyer flask. The moisture contained in the SMS was considered in the calculation of the solids' loading. The flasks with the suspensions were mixed manually at room temperature and paced in an innova® 44 incubator shaker for 1 hour set at 45ºC. After that, 30 mL of a previously prepared stock solution containing the exact amount of enzyme for achieving the required dosage was added. After that, the saccharification was run for 96 h at 170 rpm and 45ºC. Samples for sugar analysis were taken after 6, 27, 48, and 96 h. By the end of the saccharification time, the sample were centrifuged at 20 000 x g for 15 minutes to separate the hydrolysates from the residual solids. Additionally, the supernatants were vacuum filtered, and the solids were washed with water. A 1-mL sample of the hydrolysate was saved for HPLC analysis, and the remaining was stored frozen until further use beyond the current project. The sugar concentrations quantified by HPLC were used for calculating the enzymatic digestibility of pretreated cellulose.

3. Results

3.1. Characterization of the spent mushroom substrate

Most of the experiments in this project were performed with spent mushroom substrates (SMS) from the cultivation of shiitake (*L. edodes*) and oyster (*Pleurotus* spp.) mushrooms on conventional initial substrates. The SMS of shiitake and oyster mushrooms are hereafter referred to as Sh-SMS and Oy-SMS. The project also included a section on SMS from the cultivation of oyster mushroom on non-conventional initial substrates. The conventional initial substrates consisted of hardwood (either birch or oak) sawdust and wheat bran. The non-conventional substrates were based on residues from Innlandet agriculture (straw from spelt, wheat, and oat) and food processing (coffee chaff). The results of the compositional analysis of all the investigated SMS samples are presented in the following subsections.

3.1.1. SMS from conventional initial substrates

The oyster mushroom SMS had a higher cellulose content (30.6% (w/w)) than shiitake SMS (28.1%) (Fig. 3.1). The content of lignin, xylan, and arabinan (12.7%,15.8%, and 2.8% (w/w), respectively) were also higher for Oy-SMS than for Sh-SMS (6.6, 1.6, and 2.2% (w/w), respectively). Lignin content in Oy-SMS was around two-fold higher than in Sh-SMS, while xylan content was ~50% higher.

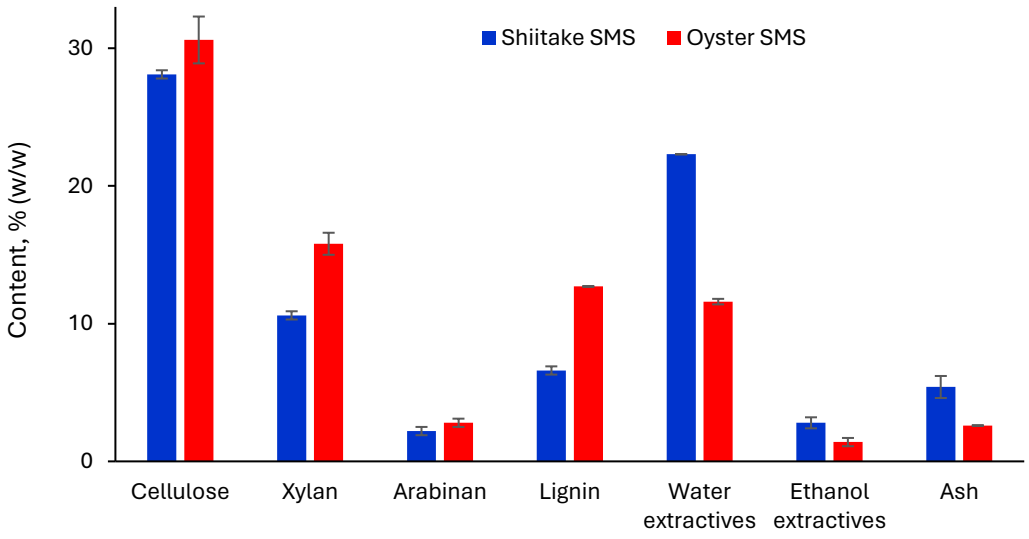

Figure 3.1. Composition of the spent mushroom substrates of shiitake and oyster mushroom grown on conventional substrates. The columns represent mean values from triplicate analyses. The standard deviations are shown as error bars.

In contrast to the trend observed for structural carbohydrates and lignin, the content of extractive compounds and mineral constituents was higher for shiitake SMS than for oyster

SMS (Fig. 3.1). For Sh-SMS, the content of water extractives was 22.3% (w/w), which was around two times higher than the value observed for Oy-SMS (11.6%). Similarly, the content of ethanol extractives was also twice as much for Sh-SMS (2.8%) compared to Oy-SMS (1.4%). The ash content of Sh-SMS (5.4%) was nearly doubled that of Oy-SMS (2.6%).

3.1.2. SMS from non-conventional initial substrates

The SMS from the cultivation of oyster mushroom on non-conventional substrates had higher content of most of the structural components than that from conventional substrate (Table 3.1). This was especially remarkable for cellulose and lignin in the SMS from the three initial substrates based on cereal straw (wheat, oat, and spelt). On the other hand, for the SMS based on oak sawdust and coffee chaff, the content of cellulose and xylan was lower than for SMS from conventional initial substrate and for the three cereal straw-based SMS.

The content of total extractives was higher for non-conventional SMS (17.3 – 23.8% (w/w)) than for the conventional one (13.0% (w/w)) (Table 3.1). The highest content of water extractives (22.5%) was observed for oat straw-based SMS, while the highest content of ethanol extractives was detected in the SMS based on oak sawdust & coffee chaff (O&C) (5.4%).

The ash content was higher for non-conventional SMS (6.6 – 13.8% (w/w)) than for the conventional one (2.6% (w/w)) (Table 3.1). The three straw-based SMS had ash content above 10% (w/w). The highest value (13.8% (w/w)) was observed for the oat straw-based SMS.

Table 3.1. Composition of the spent mushroom substrates from the cultivation of oyster mushroom on non-conventional substrates. Mean values from triplicate measurements. The standard deviations are shown in parentheses.

Component, % (w/w)	Wheat straw	Oat straw	Spelt straw	Oak sawdust & coffee chaff	Conventional (oak sawdust)
Cellulose[1]	32.9 (0.5)	41.8 (0.3)	35.8 (1.6)	25.8 (1.6)	30.6 (1.7)
Xylan	17.9 (0.5)	15.8 (0.3)	18.3 (0.7)	11.9 (1.3)	15.8 (0.8)
Lignin[2]	32.6 (3.1)	26.1 (1.6)	25.9 (0.5)	24.8 (2.1)	12.7 (<0.1)
Water extractives	15.8 (0.4)	22.5 (0.5)	19.6 (0.4)	14.8 (<0.1)	11.6 (0.2)
Ethanol extractives	1.5 (<0.1)	1.3 (0.5)	2.5 (0.4)	5.4 (0.9)	1.4 (0.3)
Ash	13.0 (<0.1)	13.8 (0.2)	11.3 (<0.1)	6.6 (<0.1)	2.6 (<0.1)

[1]Determined as glucan; [2]Determined as Klason lignin

3.2. Evaluation of the enzymatic activities of the used enzyme preparations

Three different enzyme assays, namely, filter paper, CMCase, and xylanase activities were determined for the enzyme preparations used in the analytical enzymatic saccharification study (Table 3.2). Standard methods were used for developing regression equations from the obtained standard curves of xylose and glucose concentrations.

Table 3.2. Evaluation of the enzyme preparations used in this study. Mean values from triplicate measurements. The standard deviations are shown in parentheses.

Enzyme preparation	Filter paper activity, FPU/mL	CMCase activity, U/mL	Xylanase activity, U/mL
Cellic CTec2	148.1 (4.1)	108.8 (0.6)	1 393.6 (1.2)
Trichoderma cellulases	132.5 (0.9)	4 312.1 (1.0)	618.3 (1.0)
ExpC	71.2 (0.5)	1 361.6 (0.7)	141 065.5 (0.5)

The Cellic CTec2 enzyme cocktail had the highest total cellulase activity among the tested preparations (Table 3.2). The FPA of Cellic CTec2 (around 150 FPU/mL) was slightly higher than that of *Trichoderma* cellulases (132.5 FPU/mL) and two-fold higher than that of the ExpC preparation (71.2 FPU/mL). Xylanase activity was the highest for the ExpC preparation (above 141 000 U/mL), followed by Cellic CTec2. The *Trichoderma* cellulases preparation had the highest endoglucanase activity (above 4 300 U/mL), expressed as CMCase, but it had a very low xylanase activity.

3.3. Evaluation of the enzymatic saccharification of SMS from conventional initial substrates

The enzymatic saccharification of the polysaccharides contained in the SMS was determined with analytical enzymatic saccharification, using the three enzyme preparations presented in the previous section.

3.3.1. Enzymatic saccharification of cellulose from oyster mushroom SMS

The experiment started with the evaluation of the saccharification of the cellulose contained in the oyster mushroom SMS. Oy-SMS cellulose was better saccharified with Cellic CTec2 compared to the other two preparations. The enzymatic digestibility achieved with Cellic CTec2 ranged between 46% and 60% (w/w) (Fig. 3.2-A), which was higher than the highest digestibility achieved with the *Trichoderma* cellulases preparation (42.9% (w/w)) (Fig. 3.2-B).

When the ExpC preparation was used, the values were even lower, with a maximum of only 36.8% (w/w) (Fig. 3.2-C).

In general, the cellulose digestibility was higher for the highest enzyme dosage. On the other hand, no clear correlation between the enzymatic digestibility and the loading of solids in the assay was found.

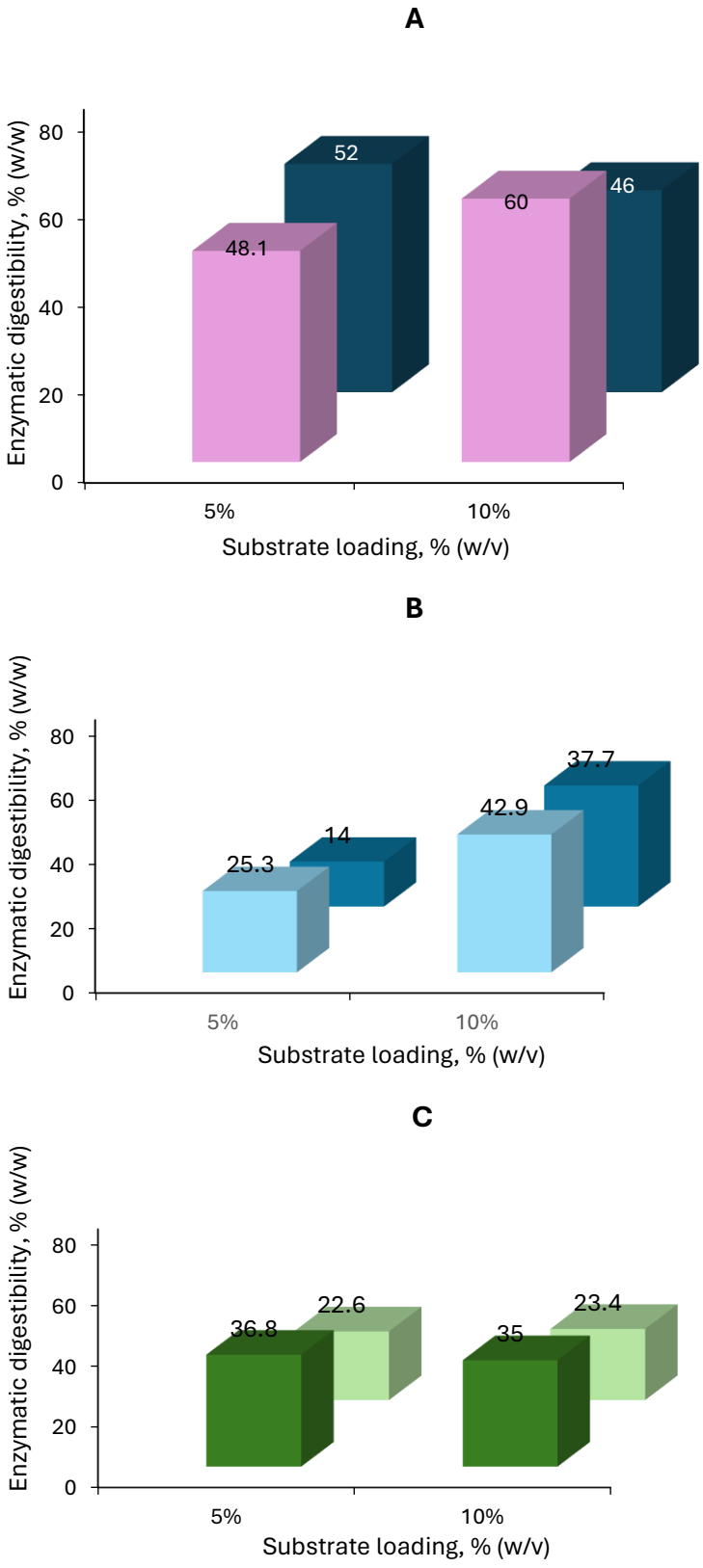

Figure 3.2. Enzymatic digestibility of cellulose contained in Oy-SMS using the Cellic CTec2 (A), *Trichoderma* cellulases (B), and ExpC (C) preparations at 25 (dark-colored columns) and 15 (light-colored columns) FPU/g.

3.3.2. Enzymatic saccharification of xylan from oyster mushroom SMS

The enzymatic saccharification of xylan contained in Oy-SMS was also evaluated. In general, a higher enzyme dosage resulted in higher digestibility. For *Trichoderma* cellulase (Fig. 3.3-B) and ExpC (Fig. 3.3-C), high substrate loads resulted in high enzymatic digestibility, whereas for Cellic CTec2 (Fig. 3.3-A), such an effect was not evident. The enzymatic saccharification with ExpC resulted in higher digestibility than with the other enzyme preparations. The highest enzymatic saccharification (32.6% (w/w)) was observed for the experiment with ExpC dosed ad 25 FPU/g and with a 10-% substrate loading (Fig. 3.3-C). The highest enzymatic digestibility achieved with Cellic CTec2 (31.7% (w/w)) was observed at the lowest enzyme dosage. (Fig. 3.3-A). That value was slightly lower than the highest observed with ExpC (Fig. 3.3-A).

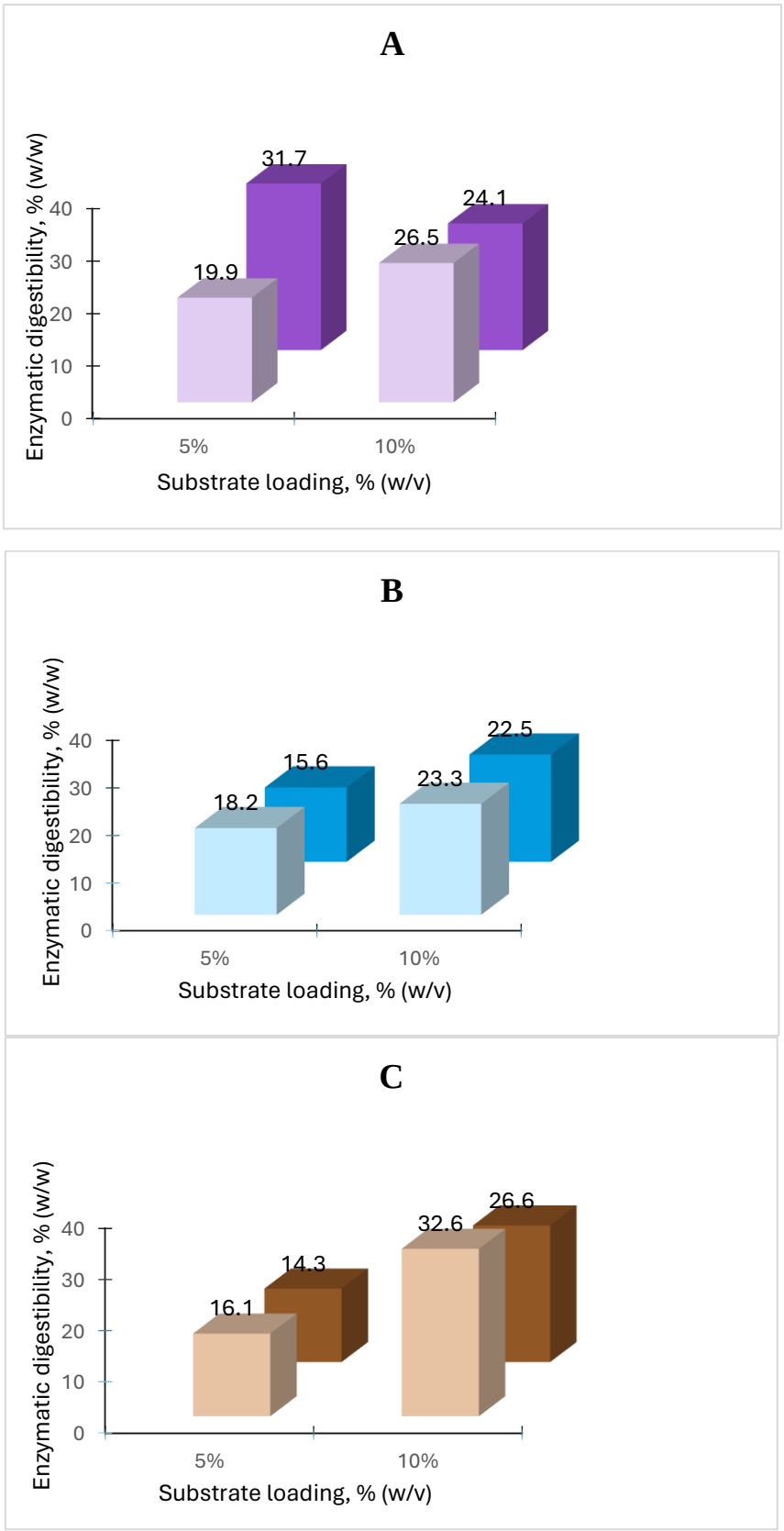

Figure 3.3. Enzymatic digestibility of xylan contained in Oy-SMS using the Cellic CTec2 (A), *Trichoderma* cellulases (B), and ExpC (C) preparations at 15 (dark-colored columns) and 25 (light-colored columns) FPU/g.

3.3.3. Enzymatic saccharification of cellulose and xylan from shiitake SMS

The enzymatic saccharification of Sh-SMS with Cellic CTec2 resulted in the overall highest digestibility of cellulose (up to 95% (w/w)) compared to the other two preparations (Fig. 3.4). The digestibility of cellulose in the experiments with ExpC was up to 77% (w/w), whereas with *Trichoderma* cellulases it was below 63% (w/w). For Cellic CTec2 and *Trichoderma* cellulases, the enzymatic digestibility of cellulose was higher than that of xylan, but for ExpC was the other way around. The enzymatic digestibility of xylan was the highest for ExpC, which resulted in values around 100%. The xylan digestibility observed with the other two preparations was not over 75% (w/w). Generally, higher dosages of enzymes and substrate loading resulted in higher digestibility of cellulose and xylan.

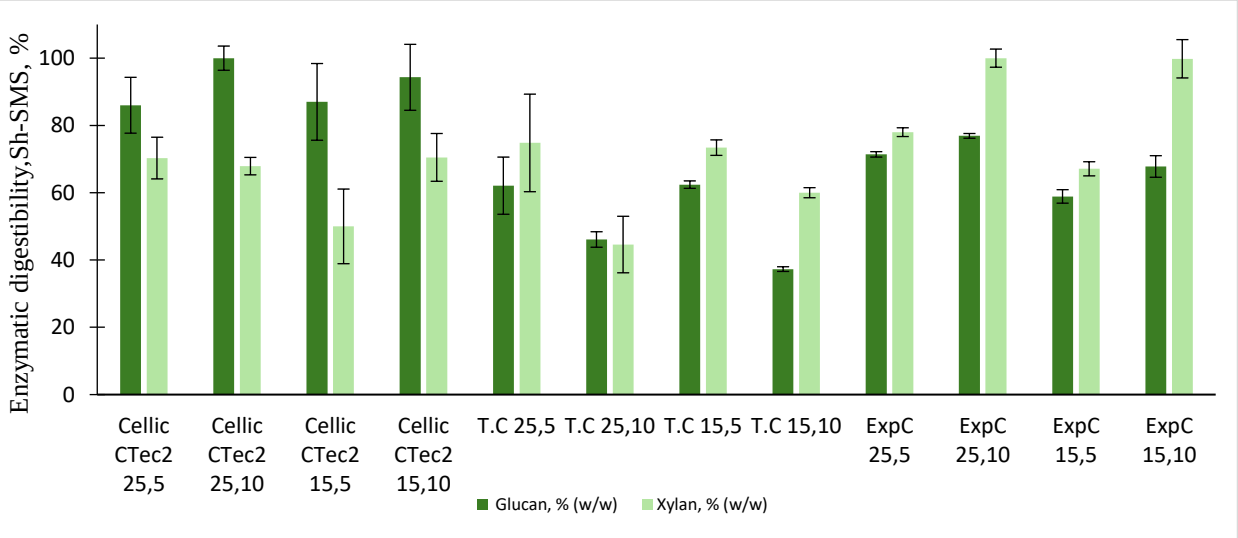

Figure 3.4. Enzymatic digestibility of cellulose (dark green) and xylan (light green) in Sh-SMS, expressed as % (w/w) using Cellic CTec2, ExpC, and *Trichoderma* cellulases (T.C) preparations at 15 and 25 FPU/g. Mean values are based on triplicate measurements.

3.3.4. Comparison of the enzymatic saccharification of SMS from shiitake and oyster mushrooms

The enzymatic digestibility of both cellulose (Fig. 3.4-A) and xylan (Fig. 3.4-B) was higher for Sh-SMS than for Oy-SMS. In the experiments with Cellic CTec 2 and ExpC, the enzymatic digestibility of cellulose contained in Sh-SMS was around two times higher than for Oy-SMS cellulose. Cellic CTec2 showed the highest digestibility of cellulose (94.3% (w/w)) (Fig. 3.4-A). The digestibility of Sh-SMS cellulose by *Trichoderma* cellulases (37.3% (w/w)) and ExpC 67.8% (w/w) was lower than by Cellic CTec2. Xylan digestibility values in Sh-SMS with the three enzyme preparations more than doubled the values observed for Oy-SMS. ExpC exhibited

highest the xylan digestibility (100%) for Sh-SMS, while Cellic CTec2 exhibited the second best value (60%). On the other hand, xylan digestibility in Oy-SMS did not differ substantially among all enzyme preparations (it was approximately 25% (w/w) for all of them). Overall, the most effective digestibility performance was observed with Cellic CTec2, with the most prominent results seen with Sh-SMS (Fig. 3.5-A, B).

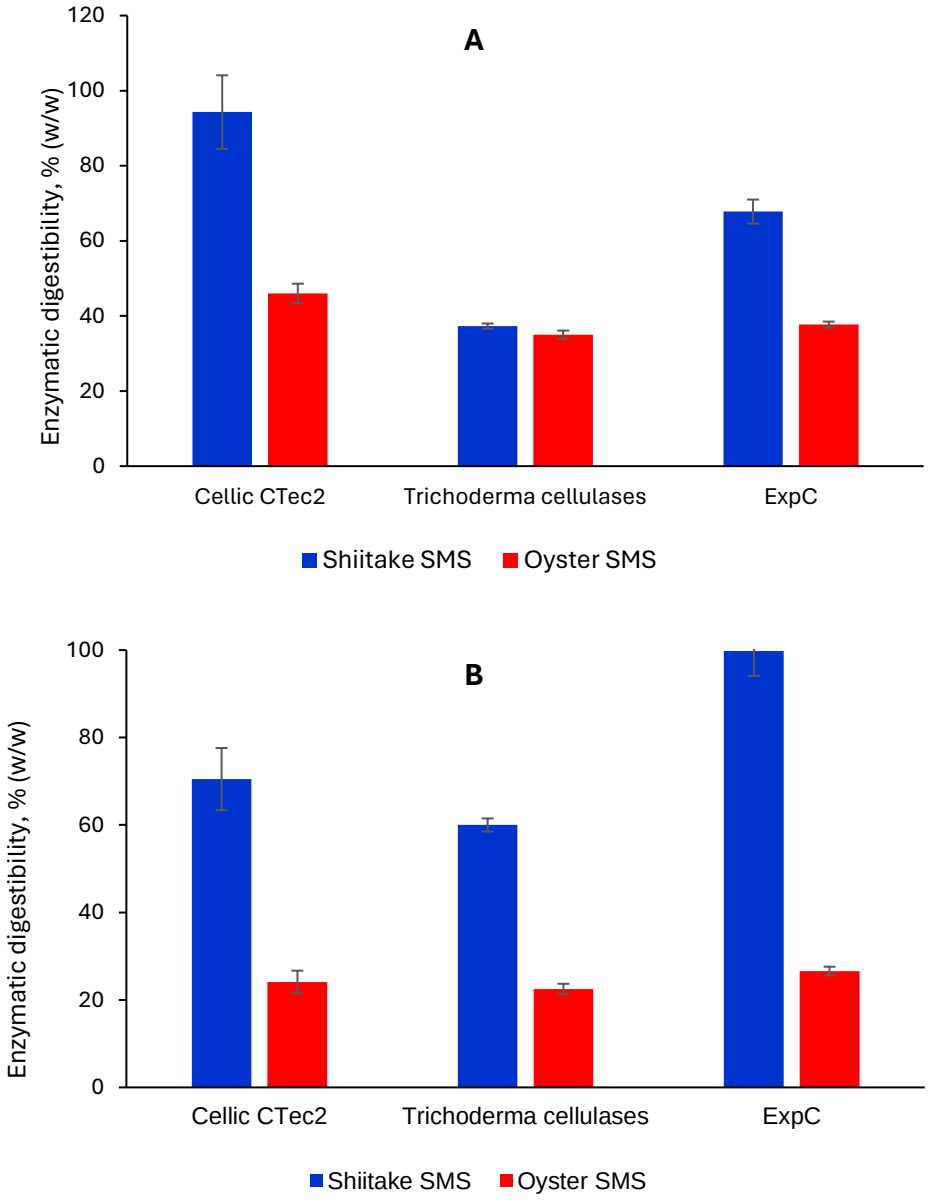

Figure 3.5. Comparison of the enzymatic digestibility of cellulose (A) and xylan (B) during saccharification of Shiitake and Oyster SMS using three enzyme preparations at a 15 FPU/g dosage and 10% (w/v) substrate loading.

3.3.5. Comparison of the enzymatic saccharification in raw and extract-free SMS

The enzymatic digestibility of raw and extract-free Sh-SMS and Oy-SMS was compared. The results showed that for Sh-SMS, the enzyme digestibility of cellulose was higher in the extract-free SMS than in the raw SMS, whereas for Oy-SMS was the other way around (Fig. 3.6). On the other hand, the enzymatic digestibility of xylan in the extract-free SMS was always higher than in raw SMS. The xylan digestibility increased by about 65% in extract-free Oy-SMS compared to raw SMS, while in Sh-SMS, the increase was approximately by 43%.

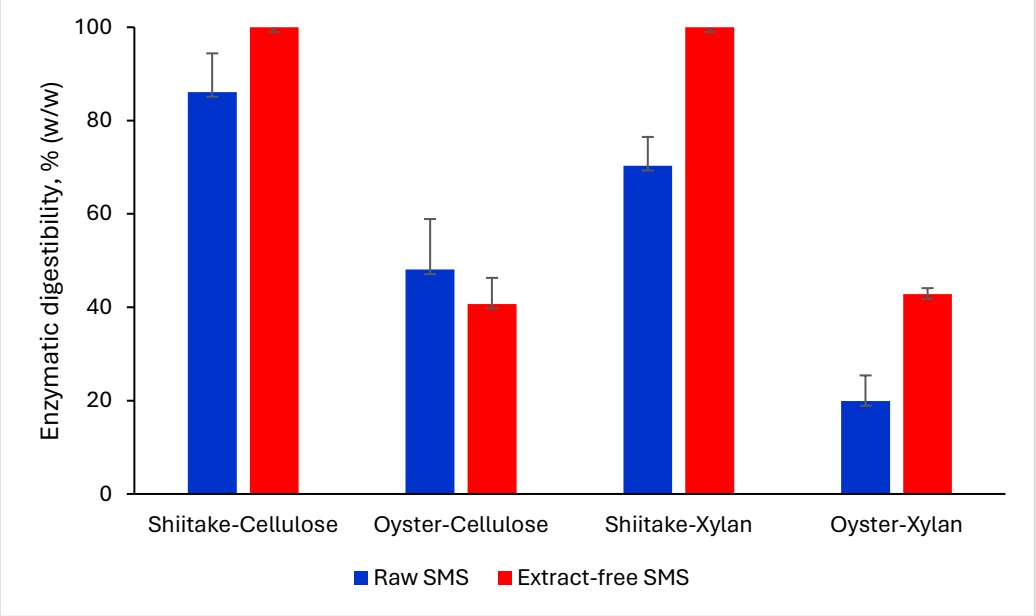

Figure 3.6. Comparison of the enzymatic digestibility of cellulose and xylan during saccharification of raw and extract-free SMS from shiitake and oyster mushroom. Cellic CTec2 was used at a 15 FPU/g dosage. The substrate loading was 5% (w/v).

3.4. Evaluation of the enzymatic saccharification of SMS from non-conventional substrates

The enzymatic saccharification of SMS from oyster mushroom cultivation on non-conventional initial substrates was evaluated. Both raw and extract-free SMS were included in the analytical enzymatic saccharification assays. The enzymatic digestibility of cellulose was generally lower in the raw "non-conventional SMS" (2.1 – 43.6% (w/w) (Fig. 3.7) than in "conventional oyster SMS" using the same enzyme preparation and conditions (52% (w/w) (Fig. 3.2-A). Only the SMS based on oak sawdust & coffee chaff (O&C) displayed an enzymatic convertibility (43.6%) that was comparable with the values observed in the SMS from conventional substrate. For the extract-free SMS, the enzymatic digestibility of cellulose was higher than for the raw

SMS, but the values were still rather moderate (5.1 – 53.4% (w/w)). The most striking difference was observed for the spelt straw-based SMS, whose convertibility was 7.9% (w/w) for the raw sample and 48.3% (w/w) for the extract-free one.

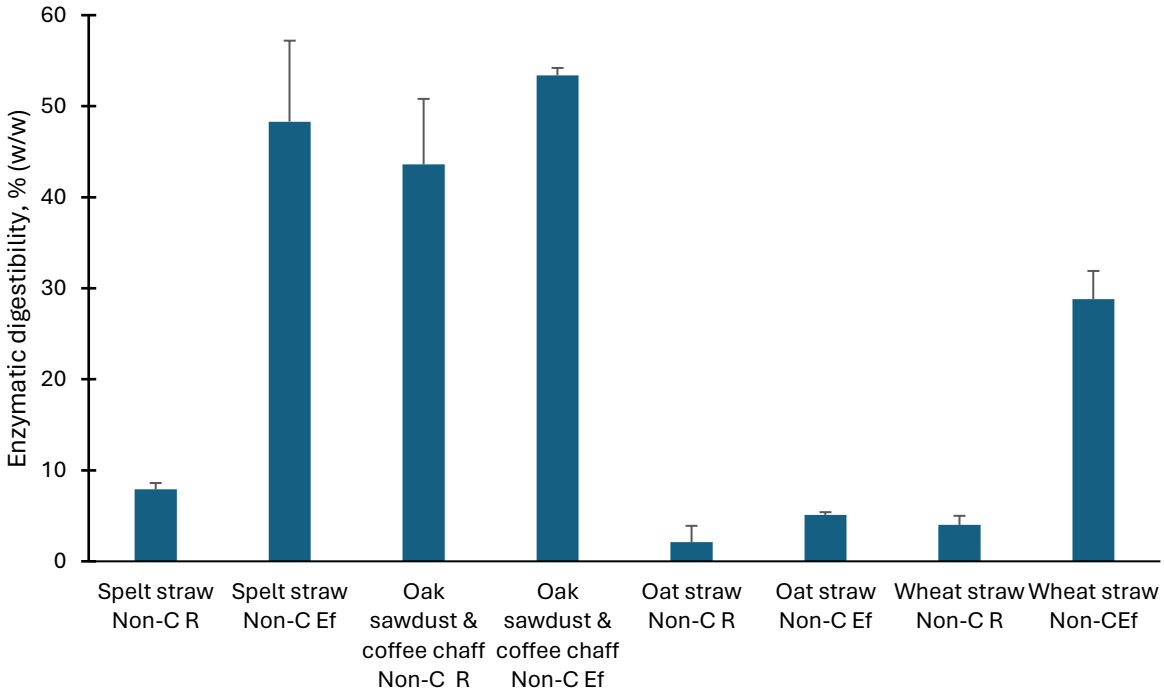

Figure 3.7. Enzymatic digestibility of cellulose in raw (R) and extract-free (Ef) SMS from oyster mushroom cultivation on non-conventional (Non-C) initial substrates. The Cellic CTec2 enzyme preparation at a 25 FPU/g dosage was used. Mean values from triplicate measurements. The error bars show standard deviations.

3.5. Hydrothermal pretreatment of oyster mushroom SMS

Hydrothermal pretreatment was conducted on oyster mushroom SMS because its enzymatic digestibility was low. The aim of the pretreatment was to enhance the susceptibility of Oy-SMS to the enzymes so that an enzymatic digestibility comparable to that of shiitake SMS could be achieved. The pretreatment was held in two runs at 175ºC using two different heating modes. One of the runs was operated non-isothermally (NI), i.e., the SMS suspension was heated to 175ºC and right after reaching that point, it was cooled down to room temperature. In the second run, a partially isothermal mode (IS) was applied, i.e., the heating and cooling periods were similar to those in the non-isothermal pretreatment, but an additional 30-min isothermal heating at 175ºC was included (Fig. 3.10).

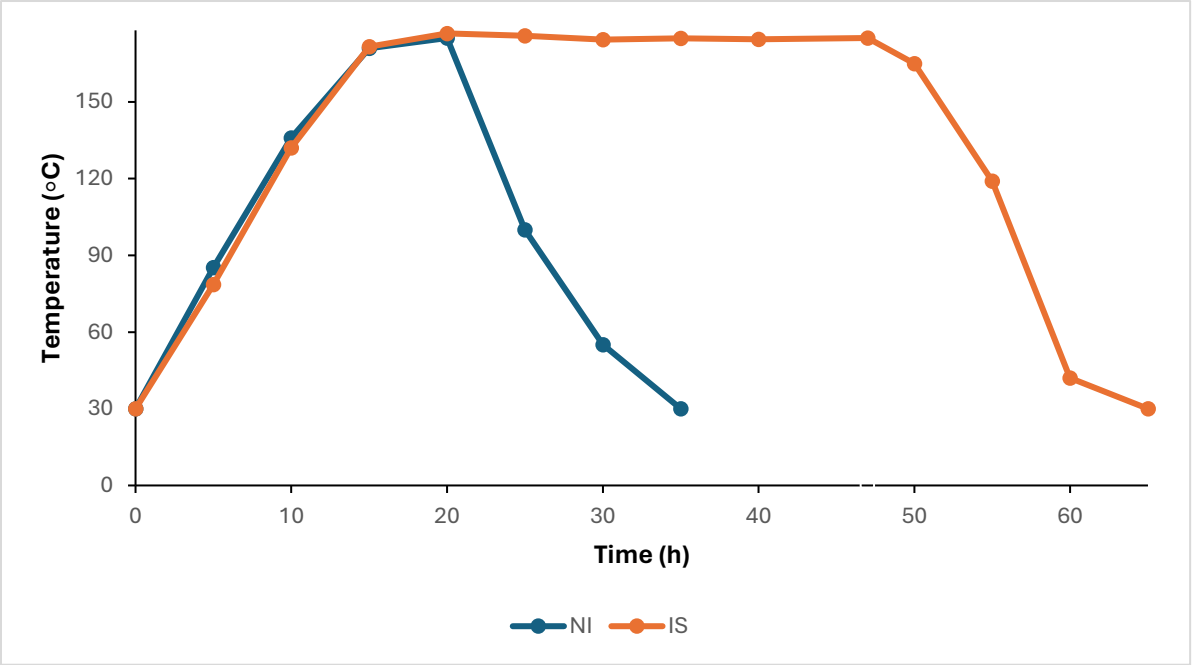

Figure 3.8. Temperature profile of the hydrothermal pretreatment of oyster mushroom SMS. Blue color, non-isothermal mode (NI); orange color, partially isothermal mode (IS).

The hydrothermal pretreatment resulted in a solids' yield of 60.0% (w/w) for the non-isothermally pretreated material and 55.7% (w/w) for the one resulting from pretreatment under partially isothermal heating mode, i.e., with a 30-min holding time at 175ºC (Table 3.3). The cellulose content in the pretreated solids was 42.1% and 46.7% (w/w), respectively, for the pretreatment under non-isothermal heating mode and for the one under partially isothermal mode. The lignin content was 24.1% and 29.7% (w/w), respectively, for the non-isothermal and partially isothermal pretreatment. No xylan or other hemicellulosic components were found in the pretreated solids from either of the pretreatments. The cellulose and lignin content were higher in the pretreated solids than in the raw SMS. Additionally, the content of cellulose and lignin in the pretreated solids increased proportionally with the length of the pretreatment. A mass balance built over the pretreatment revealed that 85-87% of the initial cellulose was recovered in the pretreated solids (Fig. 3.4).

Table 3.3. Parameters of the hydrothermal pretreatment of Oy-SMS.

Run	Temperature, ºC	Time at the temperature setpoint, min	Yield of pretreated solids, % (w/w)	Cellulose content in the pretreated solids, % (w/w)	Lignin content in the pretreated solids, % (w/w)
1	175ºC	0	60.0 (0.6)	42.1	24.1
2	175ºC	30	55.7 (0.8)	46.7	29.7

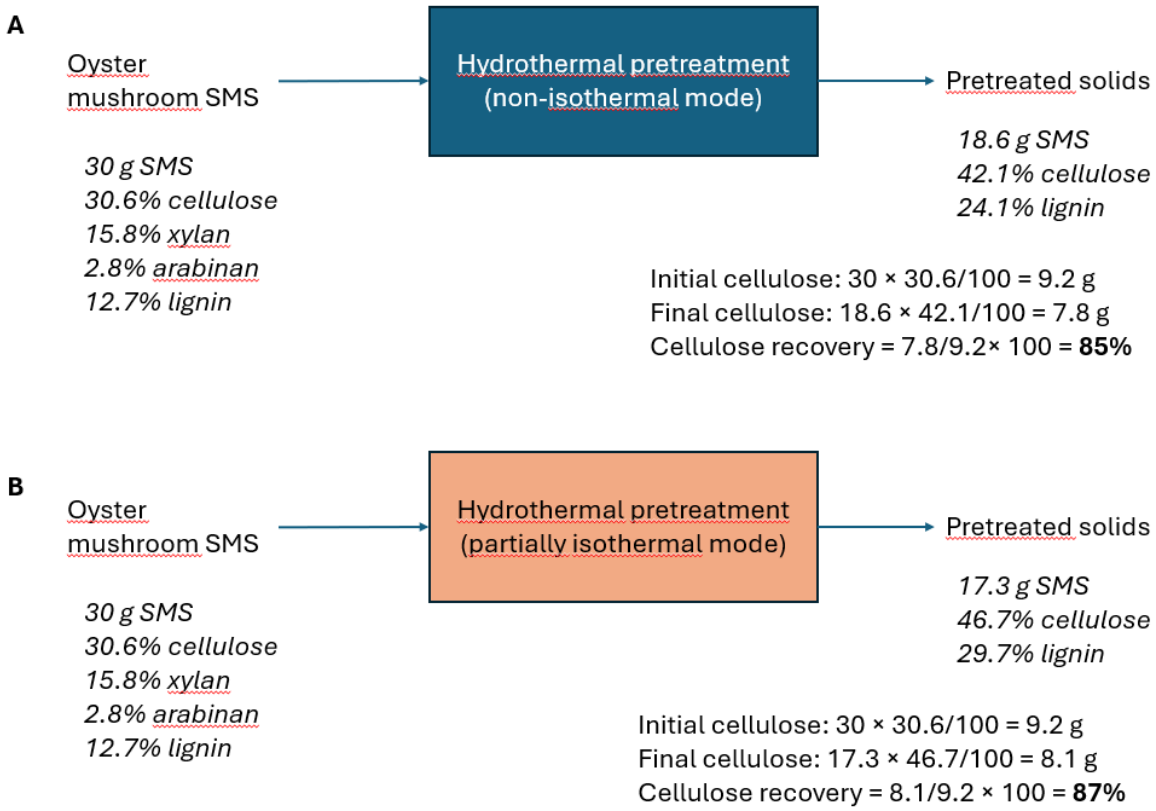

Figure 3.9. Mass balance over the hydrothermal pretreatment of oyster mushroom SMS. A, non-isothermal mode; B, partially isothermal mode.

3.6. Enzymatic saccharification of hydrothermally pretreated oyster mushroom SMS

The enzymatic saccharification of the hydrothermally pretreated Oy-SMS was evaluated and compared with that of the raw SMS. The enzyme preparation Cellic CTec2 was used. The hydrothermal pretreatment resulted in an enhanced enzymatic saccharification of cellulose (Fig. 3.10). The enzymatic digestibility of cellulose for both pretreatments ranged between 77.9 and 89.6% (w/w), which was approximately 62-84% higher than the enzymatic digestibility of the raw, i.e., not-pretreated, Oy-SMS. The enzymatic digestibility of hydrothermally pretreated Oy-SMS was comparable to that of raw shiitake SMS (Fig. 3.12).

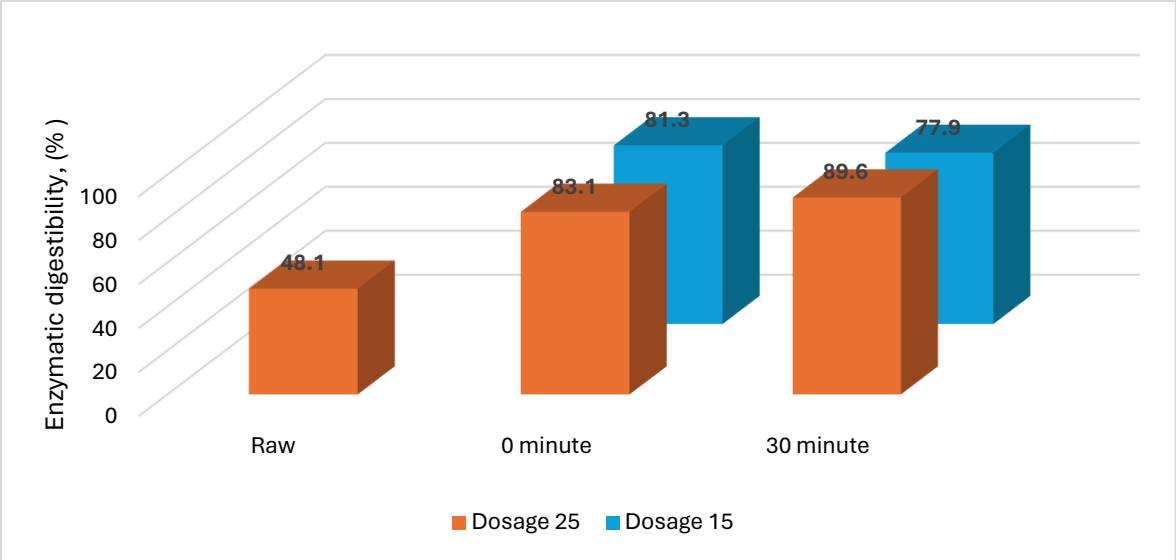

Figure 3.10. Enzymatic saccharification of cellulose in hydrothermally pretreated Oy-SMS and raw SMS using Cellic CTec2 measured as % (w/w) with enzyme preparations at 15 and 25 FPU/g. Mean values are from triplicate measurements.

The enzymatic saccharification of hydrothermally pretreated Oy-SMS was performed using two different dosages of the enzyme preparation Cellic CTec2. The effect of the enzyme dosage on saccharification varied depending on the pretreatment mode (Fig. 3.10). The highest enzymatic digestibility (89.6% (w/w)) was achieved for the pretreated solids from the partially isothermal pretreatment using the highest enzyme dosage (25 FPU/g). For the pretreated solids from the pretreatment performed under partially isothermal heating mode, the enzymatic digestibility significantly increased (p-value <0.02) with the increase of the enzyme dosage. On the other hand, for the pretreatment solids from the pretreatment under non-isothermal heating mode, no significant differences were found between the enzymatic digestibility values at two different enzyme dosages.

3.7. Assessment of the preparative enzymatic saccharification of shiitake SMS

Preparative enzymatic saccharification (PES) was performed based on the results of the analytical enzymatic saccharification (AES) experiments. The AES showed that (i) shiitake SMS has higher enzymatic digestibility than oyster mushroom SMS, (ii) Cellic CTec2 was the enzyme preparation that resulted in a better saccharification of SMS from both shiitake and oyster mushroom, and (iii) both investigated enzyme dosages (15 and 25 FPU/g) resulted in comparable enzymatic digestibility of cellulose. Based on these partial conclusions, PES was performed as an additional experiment in order to validate the results at a larger scale.

The PES experiment was performed with 60 g shiitake SMS in 2-L Erlenmeyer flasks (instead of the AES setup, which used 50 mg sample in 1.5-mL Eppendorf tubes). Cellic CTec2 at a dosage of 15 FPU/g was used. As reference, parallel saccharifications were performed with Cellic CTec3 HS, a state-of-the-art enzyme preparation developed for industrial saccharification of lignocellulosic feedstocks.

Sugar concentration was monitored during the saccharification. Glucose concentration after 6 hours was slightly higher for the Cellic CTec3 HS preparation (24.5 g/L) than for the Cellic CTec2 one (24.1 g/L) (Fig. 3.11). After that, glucose concentration was higher for Cellic CTec2 than for Cellic CTec3 HS. Xylose concentration was slightly higher for Cellic CTec2 than for Cellic CTec3 HS, but in general it was comparable for both enzyme preparations during the whole saccharification process. The samples taken at the 27-h time point, contained 33.4 and 29.1 g/L glucose for the experiments performed with, respectively, Cellic CTec2 and Cellic CTec3 HS. After that, the increase of the glucose concentration was rather marginal for both enzyme preparations. A similar trend was observed with the xylose concentration along the process. After 96 h of saccharification, hydrolysates containing 31.5 g/L glucose and 13.6 g/L xylose were obtained when Cellic CTec3 HS was used, and 35.8 g/L glucose and 15.6 g/L xylose when Cellic CTec2 was the enzyme preparation of choice.

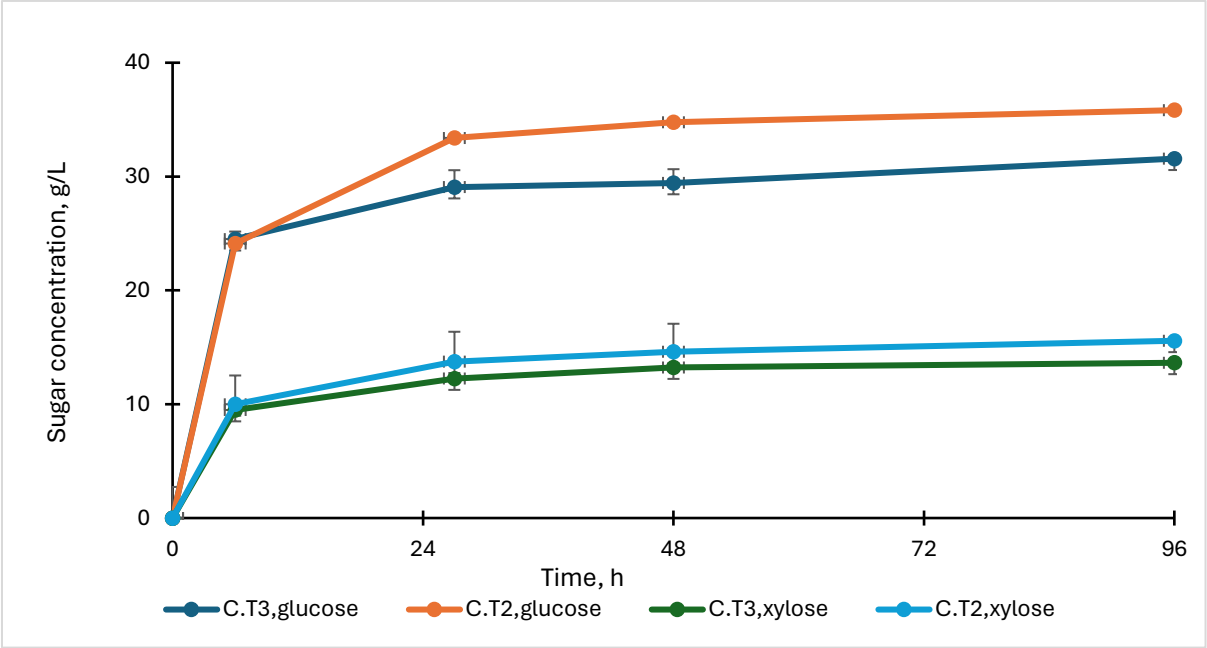

Figure 3.11. Dynamics of the sugar formation during preparative enzymatic saccharification of shiitake SMS using the enzyme preparations Cellic CTec2 (C.T 2) and Cellic CTec3 HS (C.T3)

The enzymatic saccharification generated residues containing 20.8% (w/w) cellulose, 1.4% (w/w) xylan, and 20.3% (w/w) lignin (Fig. 3.12). A mass balance over the saccharification showed that 74% of the initial cellulose and 95.6% of initial xylan were saccharified during the process (Fig. 3.12)

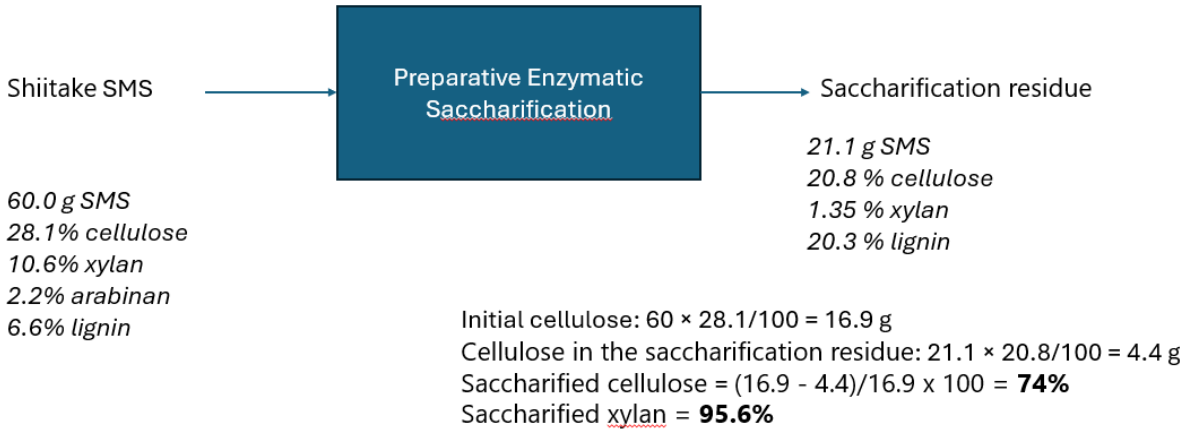

Figure 3.12. Mass balance over the preparative enzymatic saccharification of shiitake SMS.

4. Discussion

Lignocellulosic biomass is considered as a renewable resource and sustainable alternative for the production of biofuels and chemicals to reduce the reliance on non-renewable resources. Researchers are focusing on the enzymatic saccharification process to harness the potential of lignocellulosic biomass, with particular emphasis on its polysaccharide components, i.e., cellulose and hemicelluloses (Gandla et al., 2022). The enzymatic saccharification of lignocellulose polysaccharides is a part of bioconversion processes that include also microbial fermentations for producing end products of social and economic interest. A pretreatment method is required prior to the enzymatic saccharification in order to enhance the amenability of lignocellulosic feedstocks to bioconversion.

Edible fungi are cultivated in substrates consisting of lignocellulosic materials. At the end of the cultivation, a residue known as spent mushroom substrate (SMS) is generated. SMS contains cellulose and hemicelluloses that can be submitted to enzymatic saccharification for producing sugars that can further be used for producing biofuels, biopolymers, and platform chemicals. In this study, the enzymatic saccharification of polysaccharides contained in SMS of two fungal species, namely shiitake (*Lentinula edodes*) and oyster mushroom (*Pleurotus ostreatus*), was investigated. By evaluating the enzymatic saccharification of SMS polysaccharides, this study specifically examined the effectiveness of shiitake and oyster mushroom cultivation as a biological pretreatment method for preparing lignocellulosic biomass to bioconversion.

The study started by investigating the composition of oyster mushroom SMS (Oy-SMS) and shiitake SMS (Sh-SMS). The investigation revealed that cellulose, with a mass share between 28 and 31%, was the main constituent of both SMS (Fig. 3.1). That is comparable to the composition reported previously for Oy-SMS (Grover et al., 2015) and Sh-SMS (Atila, 2019; Chen at al., 2022a,b; Xiong et al., 2019). Although the cellulose content was comparable for both Oy-SMS and Sh-SMS, a substantial difference was observed in the xylan and lignin content of both SMS types: it was remarkably lower for Sh-SMS than for Oy-SMS (Fig. 3.1). The lower xylan content in Sh-SMS is most likely because shiitake degrade more the hemicelluloses than as oyster mushroom do (Li et al., 2001). Similarly, shiitake mushrooms seem to have an enhanced decomposition ability for lignin during the primordium formation stage, as noted by Villas-Bôas et al. (2003) and Atila (2019). That is the reason why Sh-SMS has a lower lignin content in comparison with Oy-SMS.

The compositional analysis also revealed that Sh-SMS contains higher levels of extractive compounds and mineral components than Oy-SMS. This might be related to phenomena occurring during fungal cultivation, but it might also be related to the initial substrate composition. The high content of extractives can be attributed to metabolites resulting from fungal cultivation and to the formation of water- and ethanol-soluble degradation products during fungal degradation of the structural polysaccharides and lignin. Apparently, there was a more thorough cultivation process for shiitake than for oyster mushroom. That can be confirmed by the previously discussed matters on xylan and lignin content. The higher ash content of Sh-SMS might be attributed to the initial substrates used for cultivation. It has been reported previously that birch-based substrate used for shiitake cultivation might contain bark, which has a high ash content that remains in the SMS after fungal cultivation (Chen et al., 2022a).

The content of extractive compounds and ash are in line with prior reports for both SMS. Klausen et al. (2023) reported almost similar levels of water extractives (13.7%), ethanol extractives (1.3%), and ash content (2.6%) for Oy-SMS from cultivation of oyster mushroom on an initial substrate composed of oak sawdust and wheat bran, which was similar to the one used for generating the SMS used in the current project. Chen et al. (2022a) reported comparable content of extractives and ash for SMS from shiitake grown on birch sawdust and wheat bran.

The study revealed a higher content of structural components in SMS from oyster mushroom cultivation on non-conventional initial substrates compared to SMS from cultivation on conventional substrates (Table 3.1). This might primarily be attributed to a poorer cultivation occurring on non-conventional substrates. This correlates well the results of a recently finished project on mushroom cultivation on substrates based on crop residues from Innlandet (Martín, 2024). There was also some influence from the initial substrate composition, as can be inferred from the high ash content of the three straw-based SMS compared with the one based on oak and chaff and with the one from conventional initial substrate. It is known that the ash content of straw from different cereals is higher than that of woody materials (Huang et al., 2016). This study included an evaluation of three enzyme preparations, two commercial (Cellic CTec2 and *Trichoderma* cellulase) and one experimental (ExpC), for their capacity to saccharify cellulose and xylan contained in Sh-SMS and Oy-SMS. As a preliminary step, the enzymatic activity of preparations was determined. The highest total cellulase activity (expressed as FPA) observed for Cellic CTec2 (Table 3.2) is in good agreement with previous reports. For example, a study by Dąbkowska et al. (2017) reported that Cellic CTec2 has the maximum hydrolysis activity

against cellulose and is commercially effective for the saccharification of lignocellulosic biomass. The preparation named *Trichoderma* cellulases has the second highest total cellulase activity, and that is well in line with the well-known ability of *Trichoderma* sp. fungi to produce significant amounts of cellulolytic enzymes, including endoglucanases and exoglucanases, which act synergistically to hydrolyze cellulose (Beldman et al., 1985). Our experiments also found that the *Trichoderma* cellulases preparation exhibited the highest endoglucanase activity (expressed as CMCase activity). This result is in agreement with a previous report showing that the CMCase activity of *Trichoderma viride* is higher than that of other cellulolytic organisms (Gashe, 1992). The evaluation of the enzyme activities also revealed that the experimental preparation ExpC had the highest xylanase activity among the tested enzyme preparations. The high xylanase activity is a consequence of the presence of several enzymes, such as β-(1,4)-xylanasse and α-(1,6)-xylanases in the ExpC crude preparation as it has been reported for other hemicellulolytic enzymes (Álvarez et al., 2016). The high xylanase activity of ExpC agrees with the high effectiveness previously observed for that preparation in the hydrolysis of hemicelluloses in softwood pulp and molasses (Khan, 2022).

The evaluation of the enzymatic saccharification of SMS polysaccharides with the three enzymatic preparations revealed that the enzymatic digestibility of cellulose was higher for Sh-SMS than for Oy-SMS (Fig. 3.5-A). The enzymatic digestibility of cellulose contained in Sh-SMS was above 90%, it was even around 100% in some runs (Fig. 3.4), whereas for Oy-SMS it was only around 46 - 60% (Fig. 3.2, 3.5). The highest cellulose digestibility for Sh-SMS than for Oy-SMS should be linked to the lignin content of both SMS. Lignin content in oy-SMS was 12.7% while it was only 6.6% in Sh-SMS (Fig. 3.1). This, in turn, is a consequence of a more thorough lignin degradation during shiitake cultivation than during the cultivation of oyster mushroom. The enzymatic saccharification of cellulose is inhibited by the adsorption of cellulases to lignin (Oliva-Taravilla et al., 2020). Therefore, a decreased lignin content in lignocellulosic biomass leads to an improved saccharification. Cellic CTec2 exhibited higher enzymatic digestibility of cellulose compared to the other two preparations. That is explained by the highest total cellulase activity in Cellic CTec2 compared with the other two preparations (Table 3.2).

The enzymatic saccharification of xylan was also better for Sh-SMS than for Oy-SMS (3.5-B). The reason for that can also be the lowest lignin content in Sh-SMS than in Oy-SMS (Fig. 3.1). The highest xylan digestibility was obtained by 100% for ExpC, likely because of its high xylanase activity compared to the other two enzyme blends (Table 3.2).

The better enzymatic saccharification of both cellulose and xylan in Sh-SMS compared with that achieved for Oy-SMS indicates that shiitake cultivation is a better pretreatment method than oyster mushrooms cultivation. Therefore, shiitake cultivation can be applied to lignocellulosic biomass as a biological pretreatment method allowing a high yield of sugars upon enzymatic saccharification. The produced sugars can be used by yeasts and bacteria in different fermentations. Hydrolysates produced from Sh-SMS under the current project are now been investigated for producing microbial oil and biopolymers.

The effect of removing the extractive compounds on the enzymatic convertibility of SMS polysaccharides was investigated. The comparison between raw and extract-free SMS in Sh-SMS demonstrated that removing extractives improved cellulose and xylan digestibility (Fig. 3.6). This agrees with a previous study by Klausen et al. (2023) on the effect of extraction on the enzymatic convertibility of cellulose contained in Oy-SMS. The observed increase in enzymatic saccharification after extraction can be attributed to removal of certain extractive compounds that might have an inhibitory effect on the cellulases (Jönsson & Martín, 2016).

The positive effect of the extraction was also observed for the Oy-SMS from cultivation on non-conventional initial substrates, where a clear increase of the enzymatic digestibility of cellulose was observed in the extract-free SMS compared to raw SMS (Fig 3.7). The explanation is the same: the enzymatic saccharification is improved because some inhibitory compounds are removed by the extraction. However, it should be mentioned that the enzymatic digestibility in the four raw non-conventional substrates was very low, which made more visible the improving effect of the extractives' removal.

Since the enzymatic saccharification of Oy-SMS was not very effective, hydrothermal pretreatment was performed to enhance its digestibility. Following hydrothermal treatment, the enzymatic digestibility of the pretreated solids increased, reaching values comparable to Sh-SMS (Fig. 3.10). The enhanced enzymatic digestibility after hydrothermal pretreatment is in agreement with previous reports using different types of lignocellulosic biomass (Martín et al., 2022). The improvement is related to different factors, such as the alteration in the lignocellulosic biomass structure and the increase of the surface area, making it more accessible to enzymatic action (Guo et al., 2014).

At the end, preparative enzymatic saccharification (PES) was performed, confirming the analytical saccharification findings. It was demonstrated that both enzyme preparations, Cellic CTec2 and Cellic CTec3 HS, resulted in comparable saccharification of cellulose and xylan

from Sh-SMS. This result was rather surprising, since it was expected that the state-of-the-art preparation Cellic CTec3 HS would result in better saccharification than Cellic CTec2. We attribute the unexpected result to the fact that the Cellic CTec3 HS preparation was received during the last weeks of this project, and a preliminary evaluation of its enzymatic activities prior to the real experiment was not possible.

5. Conclusion

The results of this study demonstrate that shiitake SMS is more susceptible than oyster mushroom SMS to enzymatic saccharification. This shows the potential of shiitake cultivation as a biological pretreatment method for bioconversion of lignocellulosic biomass.

Hydrothermal pretreatment under mild conditions is effective for enhancing the enzymatic saccharification of both cellulose and xylan contained in oyster mushroom SMS to levels comparable to those achieved for shiitake SMS.

Other valuable findings are:

- key differences were found in the chemical of shiitake SMS and oyster mushroom SMS;
- Cellic CTec2 is an effective enzyme preparation for saccharification of cellulose and xylan contained in SMS;
- the enzymatic digestibility of SMS polysaccharides is improved after removal of the extractive compounds.

This study confirms the potential of SMS as a valuable source of sugars that can be used for microbial fermentation processes for producing advanced biofuels and bio-based products.

Future research in this area should focus the study of the fermentability of SMS hydrolysates with yeast and bacteria and the suitability of the SMS saccharification residue as source of polymers for use in the materials sector. In that direction, hydrolysates produced in this study are currently being investigated for producing microbial oil using oleaginous yeasts and for producing exopolysaccharides using halotolerant bacteria, and a study on recovery of lignin and chitin from SMS saccharification residues has already started.

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