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Abstract

This work presents a new approach for lignocellulosic biomass pretreatment. The process is a sequential combination of extrusion (Ex) and semi-solid fermentation (SSF). To assess the Ex-SSF pretreatment efficiency, black spruce chips (wood residues) and corn stover (crop residues) were subjected to the process. The negative controls were the pretreatment of both residues with SSF alone without extrusion. Lignin peroxidase was the main ligninolytic enzyme contributing to the delignification in the negative controls. High lignin peroxide (LiP) activities were recorded for raw black spruce (53.7 ± 2.7 U/L) and corn stover (16.4 ± 0.8 U/L) compared to the Ex-SSF pretreated biomasses where the highest LiP activity recorded was 6.0 ± 0.3 U/L (corn residues). However, with the negative controls, only a maximum of 17% delignification was achieved for both biomasses. As for the Ex-SSF process, the pretreatments were preceded by the optimization of the extrusion (Ex) step and the semi-solid fermentation (SSF) step via experimental designs. The Ex-SSF pretreatments led to interesting results and offered cost-effective advantages compared to existing pretreatments. Biomass delignification reached 59.1% and 65.4% for black spruce and corn stover, respectively. For the analyses performed, it was found that manganese peroxidase (MnP) was the main contributor to delignification during the SSF step. MnP activity was up to 13.8 U/L for Ex-SSF pretreated black spruce, and 32.0 U/L for Ex-SSF pretreated corn stover, while the maximum MnP recorded in the negative controls was 1.4 ± 0.1 U/L. Ex-SSF pretreatment increased the cellulose crystallinity index (CrI) by 13% for black spruce and 4% for corn stover. But enzymatic digestibility of the Ex-SSF pretreated biomasses with 0.25 mL/g of enzyme led to 7.6 mg/L sugar recovery for black spruce, which is 2.3 times the raw biomass yield. The Ex-SSF pretreated corn stover led to 17.0 mg/L sugar recovery, which is a 44% improvement in sugar concentration compared to raw corn stover. However, increasing the enzyme content from 0.25 mL/g to 0.50 mg/L and 0.75 mg/L generated lower hydrolysis efficiency (the sugar recovery decreased).

Keywords: biodelignification; *Phanerochaete chrysosporium*; biomass pretreatment; fungi; extrusion; solid fermentation

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1. Introduction

The automotive and aviation industries are trying to reinvent themselves thanks to new decarbonized energy perspectives. Promising options are available today, but they have yet to be fully developed. For a long time, electric cars and their superpowered batteries have been gradually developing. Today, electric vehicles (EVs) offer the same, and sometimes even better, autonomy than conventional vehicles with a full tank of gasoline. EV production has surged in recent years since all the major automakers are now offering EV models. Some have made it their niche market with trucks, sport utility vehicles (SUV), or electric sports cars. This is why the demand for EVs is constantly growing [1]. Nevertheless, subsidies, discounts, promotions, payment by installment, loans, and other forms of incentive are all part of the EV boom. At the same time, the cost of petrol at the pump is still increasing, often by several tens of cents per liter within a few days. The reasons include geopolitical crises affecting both fuel transport costs and financial markets, resulting in excessively high costs at the pump [2]. So, modern society needs a paradigm shift in energy consumption. The aim is both to reduce energy consumption and to find decarbonized, economically viable alternatives. A sustainable lifestyle without burdening ecosystems through the indiscriminate extraction of fossil fuels would be a good step forward. However, electric batteries remain a major environmental problem, both in terms of their production (mining of rare-earth elements) and their end-of-life (recycling) [3,4]. Fortunately, a new generation of batteries with bio-sourced electrodes is under development. Another point is that the most realistic forecasts for the future predict a mix of several energies cohabiting with a preponderance towards renewable energy, rather than a sudden and definitive end to the use of fossil fuels [5]. But the race for electrification of transport has somewhat blurred the importance of biofuels, which are vital to the energy transition.

In 2024, about 1.475 billion cars worldwide with 4 or more wheels were present on the road, in the following order: 543 million in Asia, 413 million in Europe, and 358 million in North America. Of all these vehicles, only 19% were electric, i.e., around 280 million vehicles, and they are concentrated in wealthy countries [6]. This means that 81% of all cars are combustion-powered by mostly fossil fuels or renewable fuels. The advantage of biofuels is that they can be used by internal combustion vehicles without any mechanical modification to the engine. An example is a maximum of 10% bioethanol in the blend (E10) [7]. But in the 10–20% range, only a modification of the engine or fuel system is required. This places biofuels, and bioethanol in particular, in a good position.

Bioethanol production is not a new concept. The principles have been known for many decades, and the basic idea is the ethanolic fermentation of simple sugars such as glucose. But different sources of glucose must be found and available. The first bioethanol developments considered sources such as sugarcane, beet, and corn. Then, the biorefinery industry took a major step forward with the use of "energy crops": the first-generation biorefinery [8], which has been successful to this day.

As over 8 billion people are on the planet, the use of crops for energy production rather than for human consumption poses a problem. Most of these energy crops are cereals, which are the primary source of food for many people. According to the United Nations, 733 million people faced hunger in 2023; this is equivalent to one in eleven people globally [9]. Instead of using energy crops, their residues combined with forestry residues can be used as valuable alternative sources of sugars since lignocellulosic residues are made up of lignin (5–40%), cellulose (20–50%), and hemicellulose (5–35%). Cellulose is a glucose polymer based on a glucose chain, while hemicellulose is mainly a xylose polymer, and lignin is an aromatic polymer of *p*-coumaryl, coniferyl, and sinapyl alcohols.

To use agricultural and forestry residues, removing lignin to access cellulose and hemicellulose is mandatory. This step is referred to as the pretreatment. However, lignocellulose is highly recalcitrant to pretreatment, as lignin is the main obstacle. Several pretreatments have been proposed, but most of them are either not economical (too expensive) or efficient (low delignification level) depending on the raw biomass selected. Joshi et al. [10] and Woźniak et al. [11] presented an up-to-date and detailed review of the current trends in lignocellulosic biomass pretreatment.

Research on novel pretreatment approaches is attracting more and more attention. The fact is, there are still many gaps in the literature associated to lignocellulosic biomass pretreatment. While the general principles of several pretreatment methods are known (e.g., depolymerizing lignin, exposing cellulose fibers), a deeper understanding of how they affect the specific components of the biomass is crucial for optimization. This includes understanding the interactions between different pretreatments and their effect on enzyme accessibility and sugar release. Inhibitor generation is one of the main problems of most pretreatment technologies. Inhibitors are usually generated under hash pretreatment conditions, which may be necessary to break down the links in the biomass. Some authors proposed techniques to minimize or prevent inhibitor formation, but their underlying mechanism is not yet fully understood. Although they might be undesired for bioethanol production, inhibitors such as furfural and hydroxymethyl furfural can be recycled and used for high-value product formulation. On the other hand, research on efficient, inhibitor-tolerant, and cost-effective enzymes (cellulases for enzymatic hydrolysis) and yeast strains is currently under active development. The enzymatic hydrolysis step is crucial to recover the reducing sugar used for ethanolic fermentation. A low hydrolysis yield directly affects the bioethanol production and the profitability of the plant.

Several existing pretreatment technologies are very efficient in recovering the carbohydrates from the biomass, but are not scalable under their current development because of the very high investment or operation costs. Among them are ionic liquid, deep eutectic solvent, and steam explosion. Scalability is a key parameter in the selection of adequate pretreatments for commercial and industrial applications. Unfortunately, scalability studies in the literature are lacking for existing pretreatments.

If the financial aspect of the pretreatment is a determinant in its choice, the ecological aspect is nonetheless also important. As a proposed alternative to fossil fuel, the ecological aspect of the pretreatment for bioethanol production is as important as the financial aspect. Biological pretreatments are promising. They are environmentally friendly, cost-effective, and effective for lignin removal, although they take more time than other pretreatment types (physical, chemical, and physico-chemical). So, new pretreatment technology approaches involving biological pretreatment are drawing attention.

The objective of this work is to propose a novel approach for lignocellulosic biomass pretreatment involving fungal pretreatment to improve enzymatic digestibility. This study is a continuation of four consecutive previous works: (i) the use of extrusion as a pretreatment method for lignocellulosic biomass [12], (ii) pretreatment combination involving extrusion technology leading to the extrusion-biodelignification approach [13], (iii) biodelignification with fungi pretreatment [14], and (iv) an experimental study of an agricultural and a forestry biomass via extrusion pretreatment and the analysis of the extrudate [15]. The present work is an experimental study of the extrusion-biodelignification approach. A preliminary techno-economic (TEA) analysis of the proposed pretreatment is the last step. A complete TEA will be fully explored in an upcoming article.

The primary goal of the extrusion-biodelignification approach is to enhance the digestibility of carbohydrates during the enzymatic hydrolysis step. The obtained reducing sugars could be used for (but not limited to) bioethanol production. Reactive extrusion

was selected based on the advantages of extrusion technology and the characteristics of the extrudates [15]. The extrusion-biodelignification concept emerged from the idea of designing a pretreatment combination to cover the four aspects of a suitable lignocellulosic biomass pretreatment approach: efficient in terms of lignin removal, scalable, ecological, and cost-effective.

2. Materials and Methods

2.1. Raw Biomass

Two lignocellulosic residues were used in this study. First, black spruce chips (BS) were supplied by Savard et Fils sawmill (St-Ubalde, QC, Canada), while the second was corn stover (CS) provided by Agrosphere Inc (Quebec, QC, Canada). CS was a mixture of stalks, cobs, and leaves. The residues had an initial particle size between 2 and 5 mm as received. They were then ground to 1 mm particle size with a Pulverisette 15 (Frisch, Neusäß, Germany). Their elemental composition in terms of carbon, nitrogen, hydrogen, and sulfur was analyzed using a Flash 2000 CHNS/O analyzer (Thermo Scientific, Waltham, MA, USA). Their relative proportions of cellulose, hemicellulose, lignin, extractives, and ashes were determined as described in Konan et al. [15].

2.2. Extrusion

The extrusion step was carried out using a Process 11 extruder (Thermo Scientific, USA) with the specifications presented in Table 1. BS and CS were extruded under their respective optimized delignification conditions as reported in a previous study by Konan et al. [15]. The conditions for BS were 50 °C, 233 rpm (screw speed), and a biomass/water ratio of 1:1 *w*/*w*. For CS, the conditions were 65 °C, 300 rpm, and 1:1 *w*/*w* for the biomass/water ratio.

Table 1. Process 11 extruder specifications.

Parameter	Setup
Extruder type	Twin-screw
Screw type	Fully segmented, co-rotating
Screw diameter	11 mm
Max speed	1000 rpm
Torque per shaft	6 Nm
Max temperature	350 °C
Barrel zone	7 × 5 L/D electrically heated
Length (L)	82 cm
Barrel length L/D	40
Die	3 mm die *
Output	20 g to 2.5 kg/h

* All extrusion in this study was conducted without the die (it was removed to avoid clogging and excessive pressure in the barrel).

2.3. Substrate for Semi-Solid Fermentations

The extrudates (residues obtained following the extrusion step) were used directly as substrates for the semi-solid fermentation (SSF). As for the negative control, raw black spruce and raw corn stover were used for SSF without extrusion.

2.4. Fungal Strains, Inoculum, and Maintenance

The white rot fungus strain *Phanerochaete chrysosporium* A-381 (ATCC 48746) was used as a biodelignification (SSF) agent. *P. chrysosporium* has the ability to synthesize a

ligninolytic enzyme system capable of uncoupling lignin from the lignocellulosic complex. The strain was grown under sterile conditions on solid culture medium in Petri dishes (100 × 15 mm polycarbonate) for 3 days at 35 °C. The culture medium was BD 271210, composed of yeast extract (3 g), malt extract (3 g), dextrose (10 g), peptone (5 g), agar (20 g), and deionized water (1000 mL). The mycelium was harvested under sterile conditions by gently scraping the surface of the solid medium from the Petri dishes using a glass rod. The harvested mycelium was used to inoculate 50 mL of a liquid culture medium (BD 271210 without agar) contained in a 250 mL Erlenmeyer flask. This medium was placed in a shaker incubator (INFORS HT Multitron Standard, INFORS, Bottmingen, Switzerland) at 180 rpm and 35 °C for 96 h. The resulting broth was used as inoculum for both the flask fermentations (250 mL) and the 5 L fermentations.

2.5. Flask Fermentations (250 mL)

Fermentations in flasks were conducted to assess the growth conditions of the *Phanerochaete chrysosporium* strain on BS extrudates (BSE) and CS extrudate (CSE) before 5 L fermentations. The fermentations were carried out in 250 mL Erlenmeyer flasks. An experimental plan was designed for each biomass as reported in Tables 2 and 3. The experimental space was determined by preliminary trial and error. The parameters selected were based on a previous literature review by Konan et al. [14]. The parameters were temperature (°C), inoculum concentration (ml/g biomass), and nitrogen source concentration (*w*/*w* (%)). The nitrogen source was ammonium chloride (NH_4Cl), and the biomass/additive ratio was set at 1:3 (semi-solid).

Table 2. Experimental spaces.

Parameter	Black Spruce			Corn Stover		
	T	Inoculum	[NH_4Cl]	T	Inoculum	[NH_4Cl]
Unit	°C	mL/g	*w*/*w* (%)	°C	mL/g	*w*/*w* (%)
−alpha	26.6	0.16	0	26.6	0.16	0
Low level (−1)	30	0.5	2	30	0.5	2
High level (+1)	40	1.5	8	40	1.5	8
+alpha	43.4	1.84	10	43.4	1.84	10

Table 3. Experimental conditions for corn stover and black spruce.

Std	Run	Space Type	Temperature (°C)	Inoculum (mL/g)	[NH_4Cl] *w*/*w* (%)
2	1	Factorial	40	0.5	0.5
3	2	Factorial	30	1.5	0.5
13	3	Axial	35	1	0
5	4	Factorial	30	0.5	2
14	5	Axial	35	1	2.5
10	6	Axial	43.4	1	1.25
8	7	Factorial	40	1.5	2
7	8	Factorial	30	1.5	2
15	9	Center	35	1	1.25
9	10	Axial	26.6	1	1.25
1	11	Factorial	30	0.5	0.5
6	12	Factorial	40	0.5	2

Table 3. *Cont.*

Std	Run	Space Type	Temperature (°C)	Inoculum (mL/g)	[NH_4Cl] *w*/*w* (%)
12	13	Axial	35	1.8	1.25
11	14	Axial	35	0.16	1.25
4	15	Factorial	40	1.5	0.5

The biomasses were thoroughly mixed with the additive before being placed in the autoclave. For each run, 5.0 ± 0.5 g of the autoclaved biomass was introduced into a 250 mL flask with the corresponding amount of additive and inoculum. The medium was then gently mixed with a glass rod. The flask was then incubated at the selected temperature. The experimental plan was a randomized central composite design (CCD) using the software Design Expert 13. A total of 30 runs were carried out: 15 for BS and 15 for CS. During the fermentations, oxygen was supplied by the free diffusion of air into the Erlenmeyer flasks through a foam. The foam had a double role: air diffusion and prevention of microbial contamination. The pH was not monitored.

2.6. Bioreactor Fermentations (5 L Glass Tank)

Six fermentations were carried out in 5 L glass tanks, following the results of the 30 previous fermentations in flasks. The six fermentations comprised 3 for BS, including 1 fermentation with raw BS (control) and 2 with BSE produced under different fermentation conditions. The same was done for CS.

For each fermentation, 200 g of biomass was placed in the tank and autoclaved for sterilization. Then, 600 mL of ammonium chloride solution (0.5% or 2.5% *w*/*w*) was added with additional nutrients. The nutrients for 1 L consisted of KH_2PO_4 (0.6 g), $MgSO_4 \cdot 7\ H_2O$ (0.5 g), $CaCl_2 \cdot H_2O$ (0.74 g), $NH_4H_2PO_4$ (2.32 g), yeast extract (1 g), veratryl alcohol (1 mL), $CaCl \cdot 7\ H_2O$ (0.2 g), $ZnSO_4 \cdot 7\ H_2O$ (0.14 g), $FeSO_4 \cdot 7\ H_2O$ (0.5 g), $MnSO_4 \cdot H_2O_2$ (0.16 g), and $CuSO_4 \cdot H_2O_2$ (0.16 g). The batch started with the addition of the 4-day-old inoculum to the fermentation medium (300 mL or 200 mL). The substrate was stirred after colonization of the biomass by the fungus (after 4 days), and then about every 3 days thereafter. Aeration was ensured by a foam cap on top of the tank. Two of the 6 fermentations were controls (one for each raw biomass). Samples of each batch were taken every 3 days in 50 mL sterile vials for analysis. The fermentations were stopped after 24 days except for the negative controls, which were stopped after 18 days.

2.7. Enzyme Extraction

Enzyme extraction was carried out with the samples taken from the 5 L fermentations. A total of 42 samples (8 samples for each batch) were taken. Then, 5 g of the fermented substrate was transferred to a 250 mL flask with 35 mL of distilled water and placed in a shaker incubator at 37 °C and 180 rpm for 24 h. The contents were filtered with a Whatman 45 µm paper filtration device and then centrifuged at 3000 rpm and 10 °C for 10 min in a centrifuge (Allegra 6R Centrifuge, Beckman Coulter, Brea, CA, USA). The supernatant was then filtered through 22 µm PVDF syringe filters (Foxx Life Sciences, Londonderry, NH, USA), for enzymatic assays.

2.8. Enzymatic Assays

Enzymatic assays were carried out for the three main enzymes of the *Phanerochaete chrysosporium* ligninolytic system: manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac). All analyses (42 × 3 = 126) were carried out at room temperature (25 °C) using a UV–visible spectrophotometer (Nicolet IS50 FTIR Spectrometer). The substrate

used for MnP was N,N,N′,N′-tetramethyl-1,4-phenylenediamine (TMPD). Analysis of MnP activity using TMPD relies on the ability of MnP to oxidize this substrate in the presence of hydrogen peroxide (H_2O_2) and manganese (Mn^{2+}) [16,17]. The absorbance of oxidized TMPD is maximal at 611 nm. For spectrophotometric measurements, 1 mL TMPD (1 mM), 0.5 mL $MnSO_4$ (1 mM), 1 mL H_2O_2 (1 mM), and 0.3 mL enzyme extract were added to 2 mL 0.5 M sodium tartrate (pH 5). Readings were taken continuously every minute for 10 min. LiP activity was measured with veratryl alcohol at 310 nm. It is based on the oxidation of veratryl alcohol to veratraldehyde by LiP in the presence of H_2O_2 [16,18,19]. For spectrophotometric measurements, 115 µL veratryl alcohol (2 mM), 22 µL H_2O_2 (0.4 mM), and 50 µL enzyme extract were added to 2.86 mL D-tartaric acid (50 mM at pH 2.5). Laccase enzymatic activity was assayed using ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). ABTS is oxidized by laccase to form an intense blue-green cationic radical with maximum absorbance at 420 nm [16,20]. For spectrophotometric reading, 100 µL of enzyme extract was added to 2.8 mL of sodium acetate buffer solution at pH 4.5 and 100 µL of ABTS solution (5 mM). The readings were taken continuously (every minute) for 5 min. All the tests were performed in duplicate. The enzymatic activity was calculated as follows [21]:

$$Activity\ (U/l) = \frac{\Delta Abs \times V_{total}}{\varepsilon_{wavelenght} \times \Delta t \times V_{enzyme}} \times 10^6 \quad (1)$$

where ΔAbs is the absorbance variation during a time Δt; V_{total} is the total volume in ml of reaction medium for which V_{enzyme} in (mL) was added; $\varepsilon_{wavelenght}$ is the extinction coefficient ($M^{-1} \cdot cm^{-1}$) of the enzyme substrate at the wavelength at which the absorbance is measured (Beer–Lambert law). The values were 12,200 $M^{-1} \cdot cm^{-1}$ for TMPD at 611 nm, 9300 $M^{-1} \cdot cm^{-1}$ for alcohol veratryl at 310 nm, and 36,000 $M^{-1} \cdot cm^{-1}$ for ABTS at 420 nm.

2.9. Delignification

Fermented samples at 3-day intervals were oven-dried at 60 °C for 4 h before being subjected to a Soxhlet extraction process for 12 cycles to eliminate the mycelium. The solvent used was ethanol:benzene (2:1 v/v). The solid fraction after extraction was left at room temperature for drying and to evaporate the solvent. The biomass was then washed with hot tap water by filtration to remove the remaining solvent. Subsequently, 0.5 g of biomass was subjected to Klason lignin analysis. For the corn residues, an additional grinding step using a small kitchen grinder was necessary to break up the agglomerates formed during the extraction stage before Klason analysis. A 20 mL volume of 72% sulfuric acid was poured on 0.5 g of biomass and stirred regularly for 80 min. The resulting mixture was diluted to 3% H_2SO_4 with distilled water and then transferred to a digester (Digestor 2006 Tecator™ Technology, FOSS, Hilleroed, Denmark) for 3 h. The mixture was filtered using a Whatman 45 µm paper filtration device, and the solid fraction (Klason lignin) was rinsed several times with hot tap water until neutral pH (6–8). The solid fraction was then oven-dried at 105 °C and weighed. All the analyses were carried out in duplicate. The delignification rate was calculated as follows:

$$\gamma = \frac{mL_{raw} - mL_{ext}}{mL_{raw}} \times 100 \quad (2)$$

where mL_{raw} represents the mass of lignin in the raw biomass and mL_{ext} represents the mass of lignin in the extrudate.

2.10. Fourier Transform Infrared Spectroscopy (FTIR)

Samples of raw and pretreated BS and CS were scanned with FTIR equipment to investigate the effect of the pretreatment on the molecular structure of the biomasses, especially for the crystallinity index (CrI) measurement via the lateral order index (LOI) [22,23]. The measurements were performed with a Nicolet IS50 FTIR Spectrometer (Thermo Scientific, USA) equipped with a standard ATR (attenuation total reflection) crystal cell detector. Each recorded spectrum was an average of 16 consecutive scans of 4 cm^{-1} resolution from 700 to 1500 cm^{-1}.

2.11. Enzymatic Hydrolysis

Enzymatic hydrolysis tests were carried out on the raw biomasses, and all the biomasses were subjected to extrusion followed by fermentation (Ex-SSF) for 24 days. The enzymatic hydrolysis protocol was based on Danisco Us Inc [24] and Miller [25]. A total of 15 g of biomass was oven-dried at 50 °C for 24 h to obtain a dryness over 95%. The enzyme was the Accellerase Duet enzyme cocktail. Then, 12.5 g of biomass was placed in a 50 mL flask, covered with 100 mL citric buffer (0.05 M) pH 4.8, tetracycline (10 g/L in 70% ethanol), and cycloheximide (10 g/L). The mixture was placed in an incubator-agitator at 55 °C and 180 rpm for 96 h. An aliquot was taken every 24 h and placed in hot water for 5 min to stop the enzymatic reactions. The sample was then filtered and centrifuged at 3000 rpm for 10 min at 10 °C. The reducing sugars were analyzed in the supernatant using DNS (dinitrosalicylic acid) solution and a spectrophotometer [25].

3. Results and Discussion

3.1. Biochemical Composition Analysis

Lignocellulose biomass is a complex formed by cellulose, hemicellulose, and lignin in the cell wall of biomass such as wood and agricultural residues. The amount of lignocellulose and the relative proportion of its components vary from one biomass to another (softwood, hardwood, agricultural residues, grass, etc.). Lignin is the constituent limiting the enzymatic digestibility of cellulose and hemicellulose embedded in lignocellulosic biomass [26].

The pretreatment approach developed in this study was carried out on black spruce chips (BS) and corn stover (CS). Figure 1 shows the relative proportion of cellulose, hemicellulose, and lignin in both raw biomasses. BS has 60.1 ± 1.6% of holocellulose (cellulose and hemicellulose) and 28.9 ± 0.2% of lignin, while CS has 55.5 ± 1.2% of holocellulose and 18.9 ± 0.7% of lignin. Fang et al. [27] found similar results for black spruce chips, with 29% lignin and 59.7% holocellulose (glucan, xylan, mannan, galactan, and arabinan). For CS, the results are in agreement with Zhang et al. [28] who found 56.4% holocellulose and 15.3% lignin in corn residues (bark). Although the holocellulose content is higher in BS than in CS, its accessibility by enzymatic hydrolysis is technically more difficult because of its higher lignin content. There is another layer of complexity associated with the lignin recalcitrance of BS compared to CS. The former is a softwood, while the latter is an agricultural residue. Softwoods have thicker cell walls, and their lignin is structurally different from that in agricultural residues. Lignin is an aromatic polymer mainly composed of two or three units called monolignols and connected by ether (C-O-C) or carbon-carbon (C-C) bonds. These monolignols are guaiacyl (G unit), syringyl (S unit), and p-hydroxyphenyl (H unit) [29]. Softwood lignin is mainly composed of G units, while agricultural residues are mainly composed of S and H units. The G units are more recalcitrant because they are linked by strong covalent C-C bonds instead of the more cleavable β-O-4 bonds present in the other units. The S/G ratio in biomass is used as an indicator of biomass susceptibility to enzymatic hydrolysis. The lower the S/G ratio (higher the G unit) is, the more difficult the

enzymatic hydrolysis is. In the literature, an S/G ratio of 0.014 for spruce and 0.64 for corn stover were reported [26,30]. This is in contrast with hardwoods having S/G ratios of up to 2.7 [31]. In both cases, though, BS and CS are technically biomass materials for direct use in biorefineries. This is why softwood and agricultural residues are usually taken as model biomasses to assess the efficiency of pretreatments.

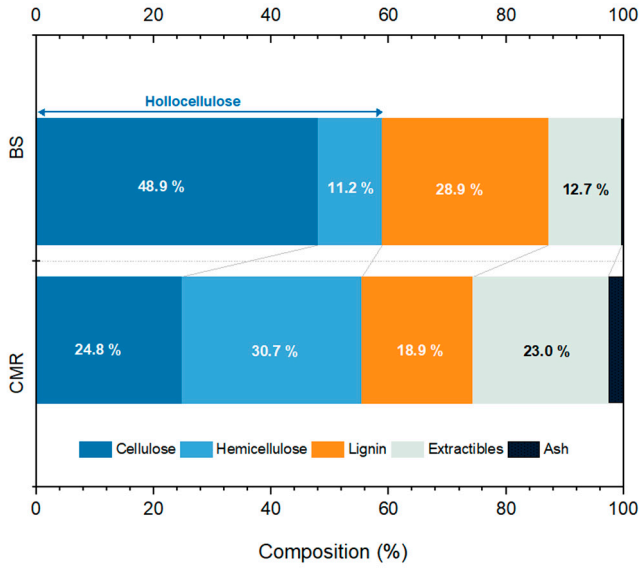

Figure 1. Composition of the raw black spruce chips (BS) and raw corn stover (CS).

3.2. Extrusion

Previous investigations on extrusion technology as a pretreatment for BS and CS confirmed that this method is effective in de-structuring lignocellulosic biomass and increasing the particle-specific surface area (see Konan et al. [15]). The analysis of the extruded biomasses for BS gave 25.3% delignification, 55 µm particle size, and 1183.3 cm^2/cm^3 specific surface area. For CS, it was 27.4% delignification, 204.6 µm, and 1569.1 cm^2/cm^3 specific surface area. These extrudates were used in this study for further biodelignification with white rot fungi to enhance the enzymatic saccharification of cellulose and hemicellulose into reducing sugars. Extrusion, unlike other pretreatments, is compatible with biodelignification because it does not generate inhibitors of microbiological activities such as furfural and hydroxymethyl furfural [32]. Furfural and hydroxymethyl furfural are compounds that are more likely to appear during most physico-chemical pretreatments due to frequent unexpected degradation of the C5 and C6 carbohydrates. In addition, the extrudates feature disrupted structures and highly increased particle specific surface area. These characteristics are well-suited for ligninolytic enzyme activities.

3.3. Flask Fermentations (250 mL)

Biodelignification consists of the removal of lignin in lignocellulosic biomass by microorganisms through ligninolytic enzymes. Only a handful of microorganisms are capable of this activity. The most remarkable ones belong to the heterogenous white rot fungi (WRF) group and are responsible for wood decaying in nature. *Phanerochaete chrysosporium* is one of the most emblematic of this group. However, WRF in general, and *Phanerochaete chrysosporium* in particular, require specific conditions for growth and to activate their enzymatic activities [33].

Flask fermentations were conducted to determine the growth conditions of *Phanerochaete chrysosporium* on extruded BS (BSE) and extruded CS (CSE). Table 4 shows the growth assessment results. Of the 15 runs carried out for each biomass, only two runs

for corn residues (CS) and six runs for black spruce (BS) showed growth of the strain. These were Run 2 and 8 for corn stover and Runs 2, 3, 4, 5, 10, and 13 for black spruce residues. As for the other runs, no microbial development was observed over 2 weeks of cultivation. However, growth rates varied from one run to the next. Strain growth on corn residues was higher and better developed than on black spruce residues (Figure 2). Strain growth on residues took the form of greenish colonization of the biomass. For corn residues, the strain was more sensitive to temperature and inoculum size, and more tolerant to the amount of additive. The only two growth conditions observed were at 30 °C and 1.5 mL/g inoculum. The amount of additive was 0.5 for the highest growth (Run 2) and 2.0 for Run 8. Thus, less additive led to higher strain growth on the biomass residues at 30 °C and 1.5 mL/g inoculum. The additive tolerance observed here was because CSE contains its own source of nitrogen. Elemental analysis of the corn residues yielded a carbon/nitrogen (C/N) ratio of 88:1. This ratio is sufficient to induce and maintain strain growth. On BSE, the strain's growth conditions were more diversified. Growth occurred between 26.6 and 35 °C; between 0.5 and 1.8 mL/g inoculum, and between 0 and 2.5% ammonium chloride concentration. No growth was observed at 40 °C and above, for either BSE or CSE, no matter the inoculum load or the ammonium chloride concentration. Yet, *Phanerochaete chrysosporium* can grow at temperature as high as 40 °C [34,35]. The water activity (a_w) is responsible for the absence of growth. In fact, a_w is crucial for *Phanerochaete chrysosporium* development. Its cells are not capable of active internal water transport [36]. Each cell obtains its required supply directly from the immediate extracellular environment. Under solid-state fermentation, there is little to no free water between the biomass particles. However, in semi-solid fermentation conditions, cells use the capillary and hygroscopic water in the biomass (Figure 3). At 40 and 46.6 °C, the capillary water evaporated after 4 to 6 h, and the hygroscopic water after 6 to 9 h. So, in less than 10 h of incubation, the cells no longer had access to water, and their development was stopped. Below 37 °C, the small amount of water available in the environment is sufficient to trigger lignin degradation, which produces additional water (H_2O) and carbon dioxide (CO_2). This explains two facts: (i) there was still growth at the lowest temperature investigated (26.6 °C); and, (ii) the optimum temperature was 30 °C. The best growth conditions occurred at this temperature for both biomasses.

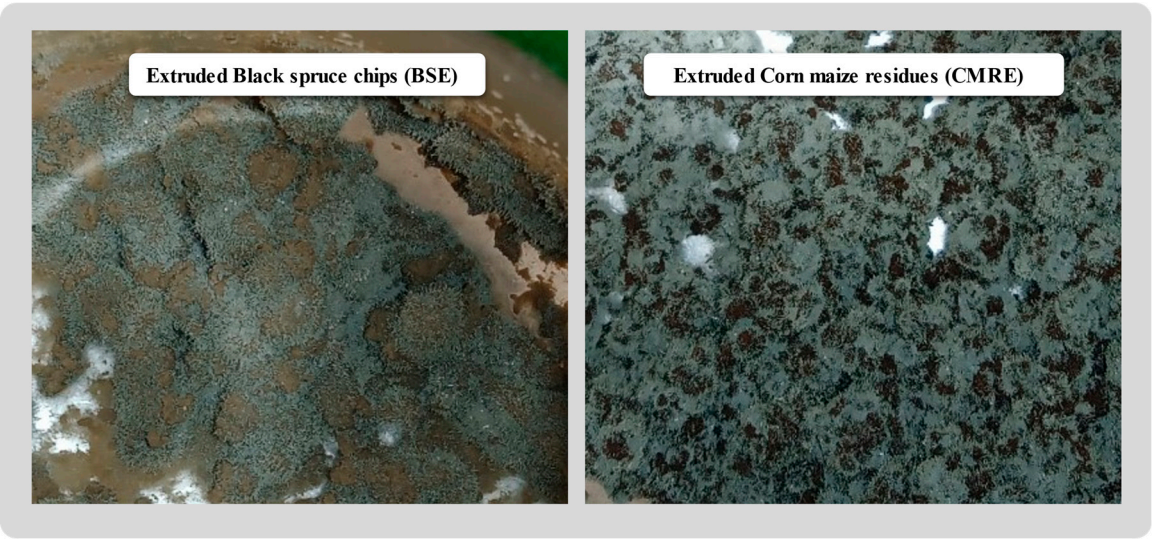

Figure 2. Colonization of extruded black spruce chips (BSE) and corn stover (CSE) by Phanerochaete chrysosporium during flask solid-state fermentation. Both BSE and CSE colonization took place under Run 2 conditions: 30 °C, 1.5 mL/g inoculum, and 0.5 *w*/*w* (%) ammonium chloride.

Table 4. Growth assessment of *Phanerochaete chrysosporium* on extruded black spruce chips (BSE) and corn stover (CSE).

Run	Conditions			BSE Growth	CRME Growth
	Temp. (°C)	Inoculum (ml/g)	[NH_4Cl] *w/w* (%)		
1	40	0.5	0.5	–	–
2	30	1.5	0.5	+++	+++
3	35	1	0	+	–
4	30	0.5	2.	++	–
5	35	1	2.5	+	–
6	43.4	1.	1.25	–	–
7	40	1.5	2	–	–
8	30	1.5	2	–	++
9	35	1	1.25	–	–
10	26.6	1	1.25	+	–
11	30	0.5	0.5	–	–
12	40	0.5	2	–	–
13	35	1.8	1.25	+	–
14	35	0.16	1.25	–	–
15	40	1.5	0.5	–	–
+++ Intense growth			++ Medium growth	+ Low growth	– No growth

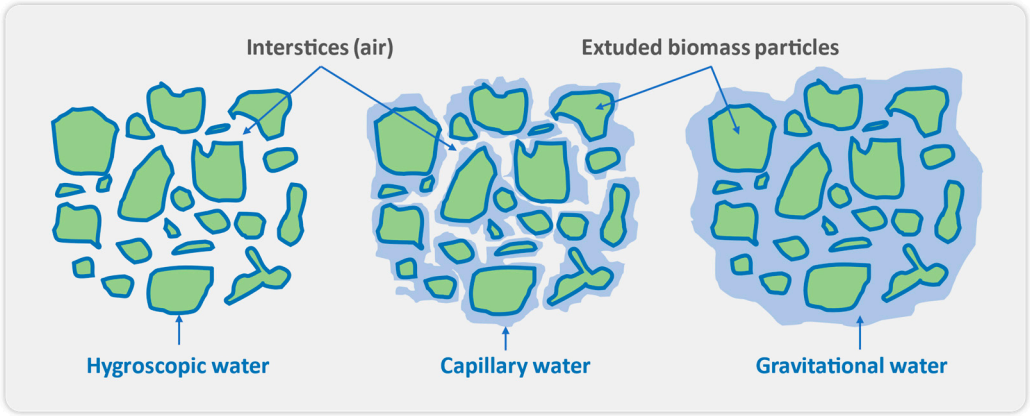

Figure 3. Type of water supply available in biomass for microbial development. Hygroscopic water (solid fermentation), capillary water (solid and semi-solid fermentation), and gravitational water (submerged fermentation).

3.4. Bioreactor Fermentations (5 L Glass Tank)

Based on the flask fermentation results, two conditions for BSE and two conditions for CSE were selected for a fermentation scale-up (40X: from 5 to 200 g). The fermentations were performed in 5 L glass tanks. The selected conditions were those of the best growth condition obtained in flasks, except for BSE where Run 4 was replaced by Run 5 (Table 2). Although Run 4 had higher mycelium development than Run 5, the analysis of biomass delignification gave 38.4% for Run 5 and 11.2% for Run 4. This is why Run 2 and Run 5 were selected for 5 L fermentation of BSE, while Run 2 and Run 8 were selected for CSE. However, one fermentation of raw BS and one fermentation of raw CS were performed as controls. Based on the water activity issues, to maximize fermentability over 24 days, the biomass/water ratio was adjusted to 0.23 ± 0.02 in the 5 L tank fermentation instead of 0.33 used during the flask fermentation. Under these conditions, the fermentations were more semi-solid fermentations (SSF) than solid-state fermentations. In addition,

basal medium components were added as nutrients in each fermentation to support rapid mycelium proliferation during the first days of fermentation. The nutrient concentration was 0.0241 g/g of biomass.

3.5. Effect of Ex-SSF Pretreatment

The goal of the bioreactor fermentation was to study the effect of the extrusion-biodelignification pretreatment on BS and CS. The focus was on the effect of different pretreatment conditions on (i) *Phanerochaete chrysosporium*'s enzymatic system, (ii) delignification rate, (iii) cellulose crystallinity, (iv) enzymatic digestibility, and (v) the role of enzyme load on enzymatic digestibility.

3.5.1. Ligninolytic Enzyme Production

Delignification of ligneous biomass by fungi is possible due to ligninolytic enzymes attacking lignin in the fibrous biomass. These enzymes are mainly laccases (Lac), manganese peroxidase (MnP), and lignin peroxidases (LiP). Contrary to most of the microbial degrading agents, *Phanerochaete chrysosporium* can release these three main enzymes. However, their secretion and relative proportion depend on the environment. Figures 4–6 present the evolution of the Lac, MnP, and LiP activities for 24 days of semi-solid fermentation (SSF) of extruded black spruce chips (BS) and corn stover (CS). All three enzymes were expressed in the controls and the extruded biomasses.

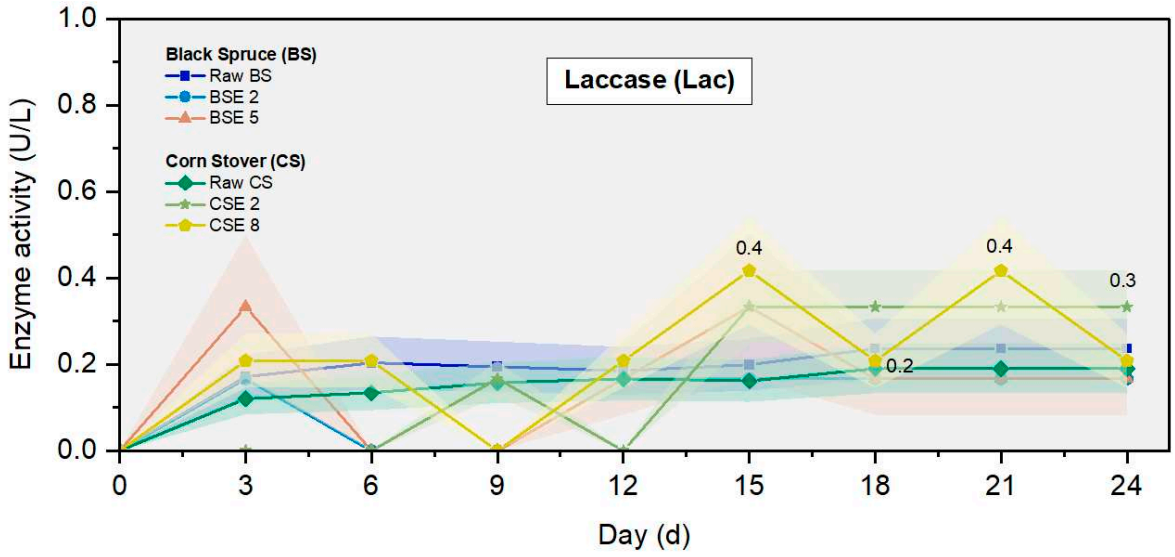

Figure 4. Evolution of laccase activity during 24 days of semi-solid fermentation (SSF) with extruded black spruce chips (BS) and corn stover (CS).

Laccase activities were very low and ranged between 0.1 and 0.2 U/L. There was no significant difference between the controls and the extruded biomasses. Laccase secretion by *Phanerocahete chrysosporium* is directly linked to the presence of copper (Cu) in the medium. Cu is not only an inducer of laccase production, but also an element of laccase structure. Laccases have four copper atoms in their structure that are important active sites for ligninolytic actions. It has been reported that for biomass with low copper content, there is little to no laccase activity from *Phanerochaete chrysosporium* [37]. The copper source in all six fermentations was from $CuSO_4$, which was at a very low concentration (0.48 mg/g of biomass). So, the lack of sufficient copper supply was responsible for the low laccase production observed in the fermentations. Copper is certainly essential in laccase production, but co-culture and supplementation of manganese oxide (IV) can also trigger laccase production. Singh et al. [38] showed that laccase production was enhanced

8.2-fold by co-culturing *Phanerochaete chrysosporium* and *Trametes versicolor* in the presence of azo dyes. In this case, the production was the result of *Phanerochaete chrysosporium* hyphal modification and its synergistic interaction with *Trametes versicolor*. As for Mn supplementation, Rodríguez et al. [39] reported a 16-fold increase in laccase production when *Phanerochate chrysosporium* was cultured on barley straw. Although techniques or additives can be used to trigger and enhance the laccase activity, economic viability must be considered.

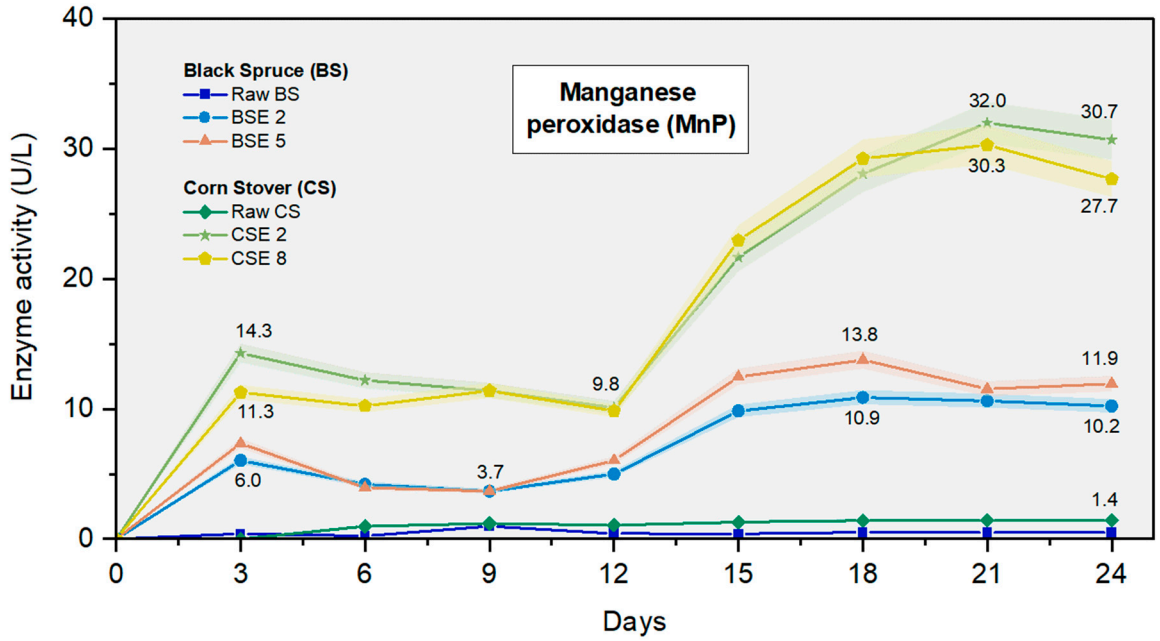

Figure 5. Evolution of manganese peroxidase activity during 24 days of semi-solid fermentation (SSF) with extruded black spruce chips (BS) and corn stover (CS).

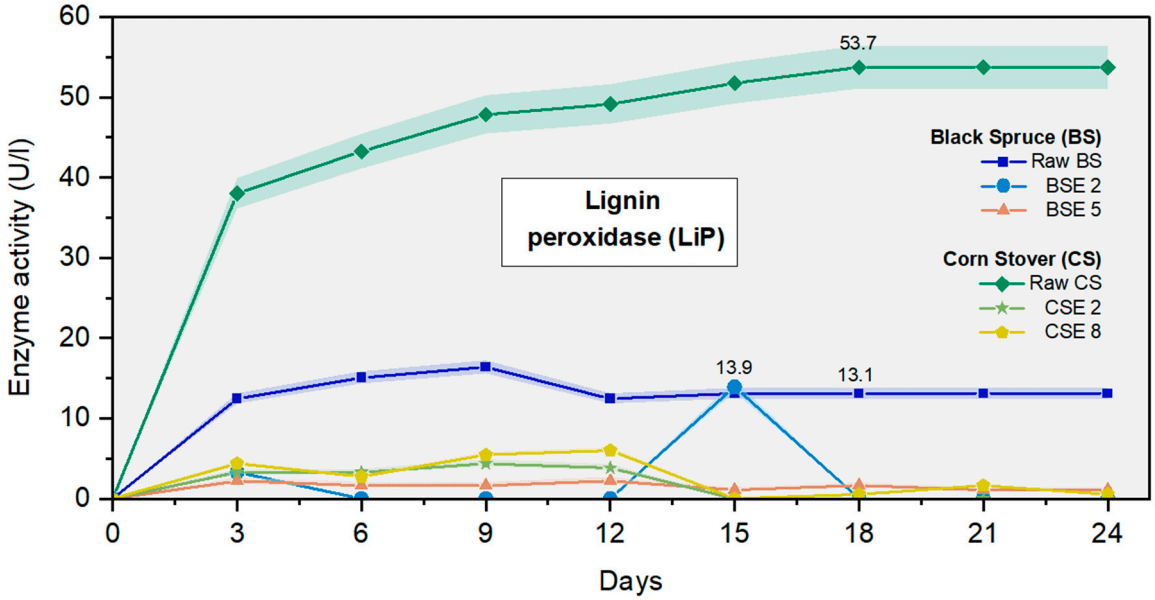

Figure 6. Evolution of lignin peroxidase activity during 24 days of semi-solid fermentation (SSF) with extruded black spruce chips (BS) and corn stover (CS).

For manganese peroxidase activity, there was a distinctive difference between both raw materials: BSE (2 and 5) and CSE (2 and 8). MnP activity was very low. The maximum MnP activity was 1.4 U/L for CSE, recorded at the end of the 24th day. However, the MnP

activities of BSE (Run 2 and 5) and CSE (Run 2 and 8) were consistently high from the 3rd day to the end, with maximum activity, respectively, of 13.8 ± 0.7 U/L (BSE 5) on day 18 and 32.0 ± 1.6 U/L (CSE 2) on day 21. These values were 28-fold and 21-fold the enzyme activity of their respective raw biomasses in less than 21 days. In general, four phases or trends can be distinguished for BSE and CSE between the 1st and 24th day. During the first days (1–3), relatively important levels of MnP activity were observed, followed by a slight decline between the 3rd and 12th day. The reason is that MnP production started in the inoculum medium before inoculation and continued until reaching a peak on the 3rd day (limited free nutrients by the 3rd day). This resulted in a decline in MnP activity. At the same time, new production of MnP was triggered by *Phanerochaete chrysosporium* to degrade lignin and find nutrients. The stress conditions due to the limited nutrients significantly increased MnP production [40]. This explains the consistent increase in MnP activity from the 9th day at a rate of 0.9 U/L per day for BSE and from the 12th day at a rate of 2.5 U/L per day for CSE. After the 18th day for BSE and the 21st day for CSE, the slight decrease observed was related to two phenomena. First, as the delignification took place in the medium thanks to the accumulation of enzymatic activity, phenolic compounds were released in the medium. These compounds from the degraded lignin are inhibitors of ligninolytic enzyme activity. Second, as the SSF was performed, a continuous drop in water activity occurred because no additional water was added during fermentation. Additionally, a decrease in available nutrients or carbon sources in the medium due to rapid consumption can increase the effect of both phenomena. These observations and explanations are consistent with the delignification rate (Section 3.5.2).

Several studies reported different ways to enhance MnP production from *P. Chrysosporium*. Ürek and Pazarlioğlu [41] obtained up to 421 U/L of MnP activity with the strain *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) by adding Mn^{2+} solution (174 µM) and Tween 80 (0.05%, *v*/*v*) to the medium. MnP uses Mn^{2+} as a mediator substrate in their mechanism of lignin degradation. Mn^{2+} is oxidized into Mn^{3+} by MnP through a series of reactions (Equations (3)–(5)). Mn^{3+} reacts with chelating agents to form powerful redox mediators capable of lignin disintegration by attacking phenolic structures. Mn^{2+} was also found to be an inducer of laccase activity in *P. chrysosporium* through a mechanism not yet fully understood. This was observed by Chen et al. [42] during a study of an antibiotic's (norfloxacin) degradation with the strain BKMF-1767 (CCTCC AF96007). In another setup, ligninolytic enzymes (including MnP) were overexpressed by 2.6 to 4-fold compared to the control activity. This setup involved a recombinant *P. chrysosporium* obtained by shock wave-induced acoustic cavitation [43]. The method requires a power source for the acoustic wave generator, which adds steps, complexity, and costs to the process. The reactions involved are as follows:

$$\mathrm{MnP} + \mathrm{H_2O_2} \rightarrow \mathrm{MnP\ I} + \mathrm{H_2O} \quad (3)$$

$$\mathrm{MnP\ I} + \mathrm{Mn^{2+}} \rightarrow \mathrm{MnP\ II} + \mathrm{Mn^{3+}} \quad (4)$$

$$\mathrm{MnP\ II} + \mathrm{Mn^{2+}} \rightarrow \mathrm{MnP} + \mathrm{Mn^{3+}} + \mathrm{H_2O} \quad (5)$$

Lignin peroxidase activity evolution was also investigated. Similarly to MnP activities, a clear trend between the raw and the extruded biomass could be observed. However, contrary to MnP, the raw biomasses had important LiP activities compared to the extruded biomass. The highest activities recorded were 53.7 ± 2.7 U/L on day 18 for CS and 16.4 ± 0.8 U/L on day 9 for BS. On the other hand, the maximum LiP activity recorded with the extrudates was 6.0 ± 0.3 U/L for CSE 8 and 13.9 ± 0.7 for BSE 2. This result highlights two facts: (i) raw CS is more suited for LiP production than raw BS, and (ii) the extruded biomasses did not favor the production of LiP, contrary to the raw biomasses. A possible explanation for the difference between LiP in raw CS and raw BS lies in the

different lignin content of these two biomasses. LiP has a specific ability to cleave β-O-4 linkages between lignin monomers [44]. β-O-4 linkages are abundant in biomass with high S/G ratios, such as CS. So, LiP production is expected to be high in such an environment, compared to a high G lignin environment where C–C links prevail. LiP is dependent on the nitrogen concentration and is expressed in a nitrogen-limited environment. Yet, extrusion did not change the C/N ratio. A previous elemental analysis of raw corn stover, black spruce chips, and their respective extruded biomasses revealed that extrusion does not change the C/N ratio of the biomass [15]. The low LiP activity in both extruded biomasses is possibly attributed to a lower content of rigid ether linkage and higher specific surface area of the extrudates particles. These combinations may significantly lower the enzymatic energy required for breaking down lignin compared to the raw biomasses where lignocellulose is intact at the beginning of the fermentation. This explanation is supported by the FTIR profile of the raw and extruded biomasses. The vibration and stretching of the functional groups, including the ether groups around 1260 cm^{-1} as well as aromatic C–O around 1024 cm^{-1}, were globally more pronounced in the extruded biomasses compared to the raw biomasses. Extrusion also significantly increased the surface potentially accessible by the enzymes: 1.5-fold (CS) and 4-fold (BSE) compared to their respective raw biomasses.

3.5.2. Delignification Rate

Biomass delignification is one of the most important parameters contributing to the improvement of biomass enzymatic digestibility for sugar recovery [45]. The biomass delignification rate was evaluated every 3 days during the bioreactor fermentations. Figures 7–10 present the results for delignification rates determined by the Klason lignin method. Based on experimental data, theoretical delignifications were assessed where Soxhlet extraction (benzene/alcohol) was less efficient in mycelium removal to accurately evaluate the experimental delignification. For some samples, the effect of mycelium presence on Klason lignin evaluation was combined with a reaction between residual benzene on the mycelium and 20 mL of 72% sulfuric acid in the presence of water and residual ethanol. The result was the sulfonation of benzene by H_2SO_4, evidenced by the whitish reaction in the medium. The relationship between biodelignification with *Phanerochaete chrysosporium* and time (in days) is a sigmoidal-like function [46]. The first phase of the curve is very slow, with an almost steady evolution of the delignification rate: this is the latency phase. No significant delignification is performed since the nutrient brought by the inoculum medium is still available as a carbon source for the fungi. The second phase is a rapid increase in the delignification rate, where lignin becomes the target for carbon source. This phase is followed by a third phase with a slight/negligible increase in the rate for various reasons, including the aging of fungal cells, CO_2 accumulation in the medium, and dropping water activity, especially if the biomass/water ratio is not adjusted over time. The Langevin function was used to build a model for BSE 2, BSE 5, CSE 2, and CSE 8 delignification evolution over time. The data, parameters, function expression, and results for each model are available as Supplementary Materials (Tables S1 and S2). When raw black spruce chips (BS) were subjected to fermentation without extrusion, a maximum delignification of 14.7 ± 0.7% was recorded after 15 days, with 12.5% delignification during the first 3 days and limited increase from the 3rd to the 15th day. As for the raw corn stover (CS) control, no delignification took place during the same period. On the other hand, when black spruce was extruded before fermentation (BSE 2 and BSE 5), the experimental delignification reached 59.1 ± 3.0% for BSE after 15 days and 65.4 ± 3.4% for CS after 18 days of fermentation. The extrusion steps contributed to 25.3% of delignification for BSE 2 and BSE 5, and 27.4% of delignification for CSE 2 and CSE 8. Globally, the first phase took place within 6 days, except for CSE 2, which was shorter (3 to 4 days). The second phase

lasted 6 days in all models, followed by the third phase beginning at day 12, except for CSE 2 at day 9. Similarly, the slopes in the second phase were 0.23 ± 0.02 except for CSE (0.33). Based on our information, there is no previous study on Ex-SSF pretreatment for biomass delignification for enzymatic digestibility and much less with black spruce chips and corn stover. However, these results are similar to those of Zhang et al. [47] on rice straw using steam explosion. The authors pretreated rice straw by steam explosion followed by solid fermentation with *P. chrysosporium*. The delignification results were a sigmoidal function with a 0.25 slope and a maximum delignification of 58% after 12 days under optimized conditions including supplementation with 0.3% Tween 80. Moreover, some studies have reported biodelignification with *Phanerochaete chrysosporium* on different type of biomass residues (agricultural and forestry), with varying success depending on the biomass and the fermentation conditions. In order to enhance the delignification of paddy straw and pine needle biomass, a work combined weak acid pretreatments (alkali, peroxide, etc.) and fungal pretreatments [48]. Up to 47.1% delignification was achieved after 28 days of fermentation in bags at 28 °C with *Phanerochaete chrysosporium* as the biodelignification agent. At the same temperature (28 °C) and with the same fungi species (*P. chrysosporium*), several conditions were studied: pretreatments of 5 g of apple pomace for 7 days [49], 20 g of switchgrass for 35 days [50], 2.5 g of wheat straw for 15 days [51], and 10 g of a blend of Olive pulp and wheat straw for 84 days [52]. The different pretreatments yielded 19.8%, 24.1%, 45.6%, and 60% delignification, respectively. These pretreatments used less than 50 g of biomass and used chemicals, contrary to this study with 200 g and without chemicals.

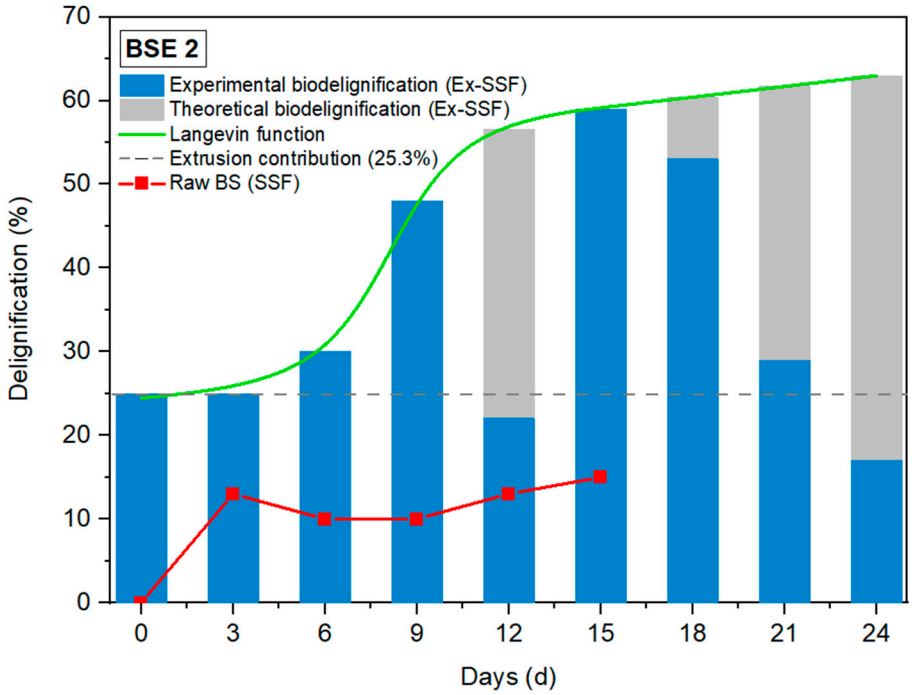

Figure 7. Biomass delignification rate during the tank fermentation of black spruce chips in Run 2 conditions. The red line with square symbols is the control fermentation with raw black spruce chips without extrusion (BS).

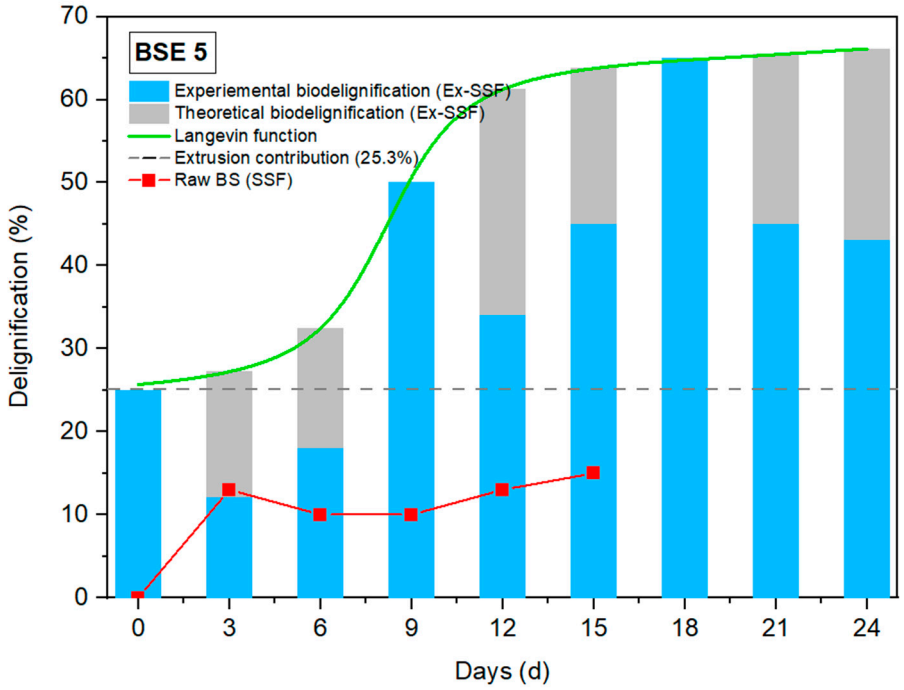

Figure 8. Biomass delignification rate during the tank fermentation of black spruce chips in Run 5 conditions. The red line with square symbols is the control fermentation with raw black spruce chips without extrusion (BS).

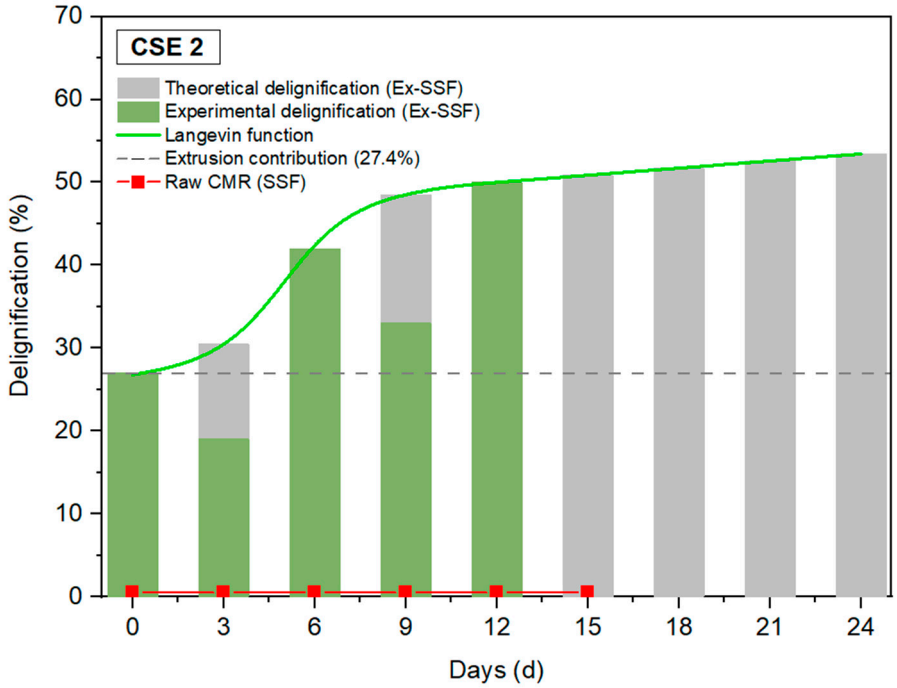

Figure 9. Biomass delignification rate during the tank fermentation of corn stover in Run 2 conditions. The red line with square symbols is the control fermentation with raw corn stover without extrusion (CS).

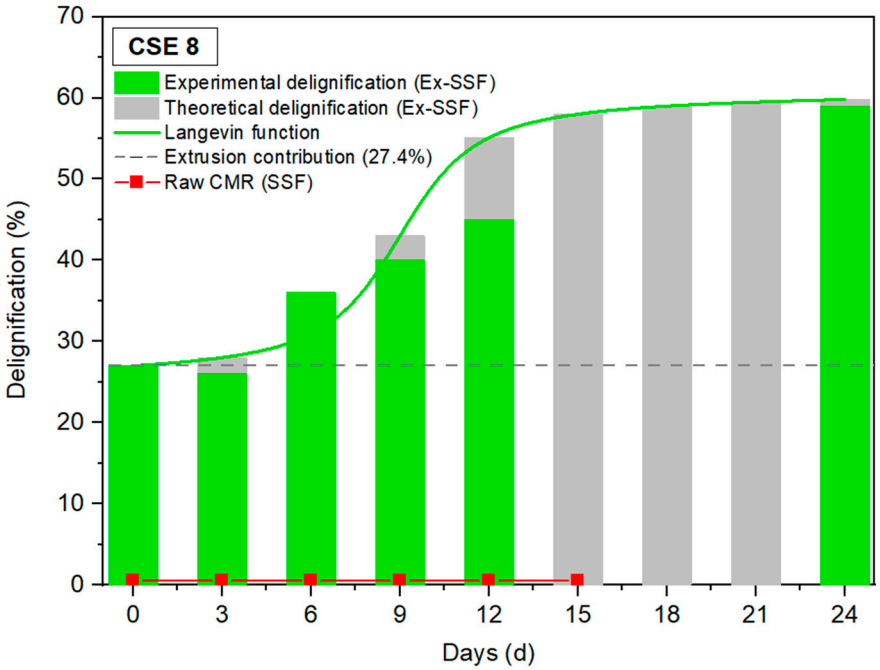

Figure 10. Biomass delignification rate during the tank fermentation of corn stover in Run 8 conditions. The red line with square symbols is the control fermentation with raw corn stover without extrusion (CS).

3.5.3. Crystallinity Index

Fourier transform infrared spectroscopy (FTIR) has been widely used to determine the crystallinity index (CrI) in biomass since the early work of Vydrina et al. [53]. It is based on the method proposed by Hurtubise and Krassig [54] and Nelson and O'Connor [55] on the lateral order index (LOI) taken equivalent to CrI. CrI is calculated as the ratio of the peaks recorded at 1427 cm^{-1} (A_{1427}) and 898 cm^{-1} (A_{898}). CrI, similarly to biomass delignification, plays a major role in enzymatic saccharification. Theoretically, amorphic regions give better accessibility to enzymes for saccharification [56]. In the literature, the crystallinity index is usually defined as the ratio of crystalline cellulose to amorphous cellulose. If this is true for pure cellulose, it is not the case for lignocellulose. Actually, the amorphous region measured by the A_{898} peak is not only available for amorphous cellulose, but also for hemicellulose and lignin [57].

Figure 11 shows the A_{1427} and A_{898} peak locations for the raw biomasses (untreated and pretreated by the Ex-SSF approach), while Table 5 presents the crystallinity index. Untreated black spruce had a crystallinity index of 54.8%, while untreated corn stover had an index of 46.9%. Black spruce contained almost twice the amount of cellulose found in corn stover (48.9% vs. 24.8%) and was expected to have a higher CrI than CS. However, the results show that the Ex-SSF pretreatment significantly increased the CrI for BS by 13.0% (BS2) and 9.8% (BS5) compared to CS with only 4.0% (CSE 2) and 2.7% (CSE 8). These results suggest that a decrease in the amorphous region in the biomass occurred. The observed increase in the crystallinity index following the pretreatment is primarily attributed to the selective removal of the amorphous regions, mainly lignin and hemicellulose, while preserving the crystalline cellulose domains. From a molecular standpoint, lignin is covalently linked to hemicellulose and cellulose via ester and ether bonds, such as *p*-coumaryl ester linkages and β-O-4 aryl ester bonds [58]. During the pretreatment, mechanical shear and microbial enzymatic activity target these linkages, focusing on cleaving lignin carbohydrate complexes (LCC), thus facilitating the delignification process. The disruption of these molecular associations loosens the lignin matrix, enhancing lignin solubilization

and increasing cellulose accessibility. As lignin and hemicellulose are progressively solubilized, the crystalline cellulose content in the overall biomass matrix increases, leading to a higher CrI [59]. This is not necessarily due to a true increase in crystallinity, but rather to the relative enrichment of crystalline regions as amorphous structures are eliminated. Moreover, the pretreatment may remove the hemicellulose sheathing around cellulose fibrils, allowing a more compact packing of cellulose chains and slight recrystallisation, further contributing to CrI enhancement. A higher CrI supports the hypothesis of effective lignin removal without significant cellulose degradation. These structural modifications are essential for subsequent enzymatic hydrolysis, as they enhance cellulose accessibility and surface area, thereby improving saccharification efficiency [60]. Delignification analysis and CrI evolution serve as useful indicators of pretreatment efficiency and the extent of biomass deconstruction at the molecular level.

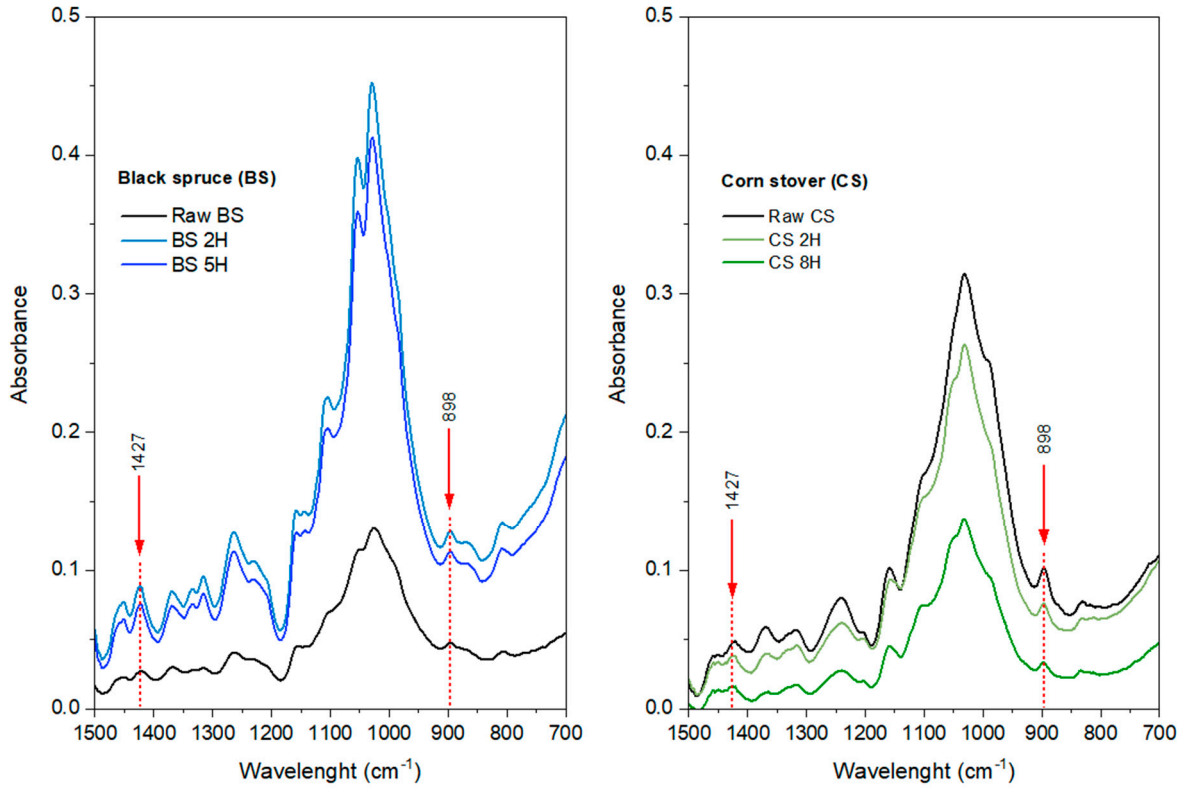

Figure 11. FTIR spectra of the raw and Ex-SSF pretreated black spruce chips (BS) and corn stover (CS).

Table 5. Crystallinity index of the samples.

Parameter	Black Spruce Chips (BS)			Corn Stover (CS)		
	Raw BS	BSE 2	BSE 5	Raw CS	CSE 2	CSE 8
A_{1427}	0.02599	0.08621	0.07261	0.04692	0.03778	0.01599
A_{893}	0.04744	0.12715	0.11247	0.10006	0.07431	0.03226
CrI (%)	54.7%	67.8%	64.5%	46.8%	50.8%	49.5%

CrI increasing after pretreatment has been reported by several studies [61–65]. For example, Fatria et al. [65] pretreated bamboo by microwave followed by fungus (*Trametes versicolor*). For all the pretreatment conditions, they recorded an LOI increase from 1.2 to 3.2%. Chinwatpaiboon et al. [61] pretreated Napier grass with *Clostridium beijerinckii* JCM 8026 and recorded a 38% crystallinity index increase after acid pretreatment with H_2SO_4 and a 41% increase after alkali pretreatment with NaOH. The high intensity of the bands in

the pretreated BS biomass compared to the raw biomass confirmed the disruptive effect of the Ex-SSF pretreatment on BS. The functional groups could vibrate and stretch more freely than in the rigid structure of the raw biomass.

3.5.4. Enzymatic Digestibility

The effect of the Ex-SSF pretreatment on decreasing the sugar yield after enzymatic hydrolysis was investigated over a period of 96 h for black spruce chips and corn stover. Figure 12 presents the evolution of the sugar concentration as a function of time. After 96 h, the sugar concentration was reduced by more than 2 times for BS pretreated with Ex-SSF compared to the unpretreated BS. The sugar concentration was 7.6 $\pm$ 0.4 g/L for BSE 2 and 7.1 $\pm$ 0.4 g/L for BSE 5, while it was 3.3 $\pm$ 0.2 g/L for the raw BS. For CS, the Ex-SSF pretreatment improved the saccharification by 44% (CSE 2) and 36% (CSE 8). The trend of the pretreated biomass curves suggests that the enzymatic saccharification was still in progress and better yields could be obtained if the tests were not stopped. Run 2 gave the best sugar concentration for both biomasses (BS and CS). The conditions were 30 °C for fermentation, 0.5 mL/g for inoculum, and 0.5% (*w*/*w*) for ammonium chloride. This result is in agreement with the observations during flask fermentation, where Run 2 gave the best mycelium development for both BS and CS (Table 4 and Figure 2). Additionally, BSE 5 and BSE 2 had similar sugar concentration reductions, as well as CSE 8 and CSE 2. This is consistent with their delignification rate and crystallinity index: two key parameters of enzymatic hydrolysis [66]. BSE 5 and BSE 2 had only a 3.2% difference in biodelignification (models) and 3.2% difference in CrI. The same conclusion applies for CSE 2 and CSE 8, with 6.4% biodelignification (models) and 1.3% CrI differences, respectively. However, enzymatic hydrolysis with corn stover (pretreated or not) achieved 2.2 times higher sugar reduction than black spruce hydrolysis even though CS had 2 times less cellulose content. In fact, reducing sugars are from holocellulose (cellulose + hemicellulose). The holocellulose content in both the BS and CS biomasses was close, at 60.1% and 55.5%, respectively. However, black spruce had almost twice the lignin content (29.8%) of corn stover (18.9%) (see Figure 1). Therefore, the sugar reduction recorded for both biomasses is aligned with the predictions and confirms that lignin is the main obstacle to enzymatic hydrolysis efficiency. This also justifies the relevance of the Ex-SSF pretreatment.

3.5.5. Effect of Enzyme Load on Enzymatic Digestibility

Several studies investigated the effect of biomass load on enzymatic hydrolysis of pretreated biomass. It was shown that increasing the biomass content led to lower efficiency of the enzymatic digestibility. The reasons are manyfold: poor mass transfer of cellulolytic enzymes, formed sugar inhibition effect, higher substrate viscosity, etc. [67–69]. To optimize the enzymatic hydrolysis in this study, the enzyme content was increased from 0.25 mL/g of biomass to 0.5 mL/g and 0.75 mL/g. For this investigation, BSE 2 and CSE 2 were selected, as they gave the best hydrolysis yield with 0.25 mL/g load. Raw biomass of black spruce and corn stover was also subjected to 0.5 mL/g and 0.75 mL/g enzyme content for the controls. The sugar reduction was again recorded for 96 h, and Figure 13 presents the results. No significant increase in raw BS sugar reduction yield (0.7% increase) occurred. On the other hand, increasing the enzyme content negatively affected the sugar yield of the Ex-SSF pretreated black spruce (BSE 2). The concentration dropped from 7.6 g/L with 0.25 mL/g enzyme to 5.9 g/L and 5.1 g/L with 0.50 mL/g and 0.75 mL/g enzyme, respectively (33% reduction). The same trend was observed for raw corn stover (CS) and pretreated corn stover (CSE 2). A 47.5% reduction for raw CS was recorded, and up to 70.6% for CSE 2. No significant differences between the 0.5 mL/g and 0.75 mL/g values were observed. Contrary to BS, where the pretreated biomass had higher sugar reduction

concentration compared to the raw biomass, the pretreated corn stover (CSE 2) exhibited less sugar yield than the raw biomass (raw CS). This result suggests that the enzyme content is very important to maximize the enzymatic hydrolysis. For this conclusion, it can be assumed that better hydrolysis yields than those with 0.25 mL/g enzyme content can be obtained if the enzyme content is optimized. However, this was not the case in this study. The reduction in sugar concentration is not the result of end-product inhibition, in which case, the sugar accumulation would have been rapid during the first 24 h followed by a rapid stagnation (plateau). Rather, the results suggest an enzyme saturation effect.

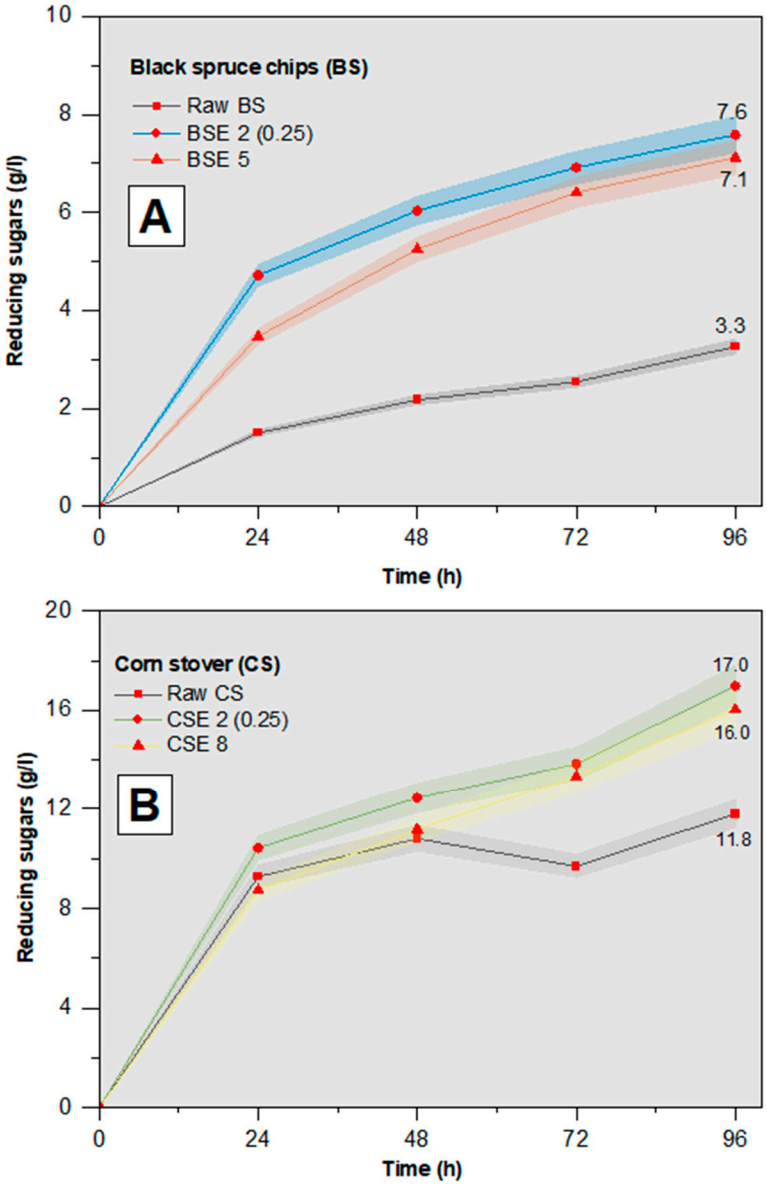

Figure 12. Reducing sugar concentration as a function of hydrolysis time with an enzyme content of 0.25 mL/g of biomass. (**A**) presents the results for raw black spruce (BS) and black spruce pretreated in Run 2 (BSE 2) and Run 5 (BSE 5), while (**B**) presents the results for raw corn stover (CS) and pretreated corn stover in Run 2 (CSE 2) and Run 8 (CSE 8).

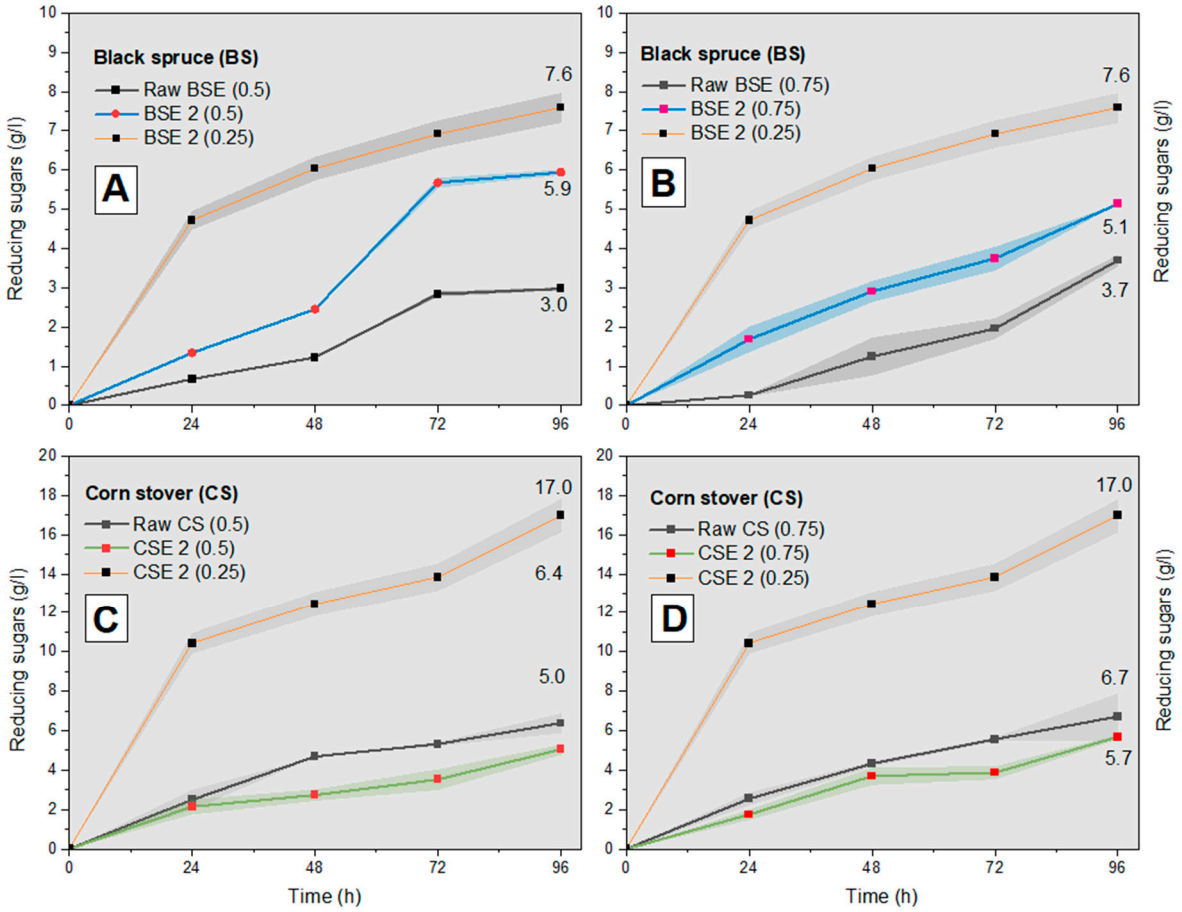

Figure 13. Sugar concentration reduction as a function of hydrolysis time with an enzyme content of 0.50 and 0.75 mL/g of biomass. (**A**,**B**) present the evolution of sugar concentration for raw BS and pretreated black spruce in Run 2 (BSE 2): (**A**) for 0.50 mL/g and (**B**) for 0.75 mL/g, while (**C**,**D**) present the evolution of sugar concentration for raw CS and pretreated CS in Run 2 (CSE 2) with (**C**) for 0.50 mL/g and (**D**) for 0.75 mL/g.

Accellerase Duet is a consortium of different enzymes including endoglucanase, β-glucosidase, and xylanase [70]. Each of them has a particular substrate and attack site in the biomass. When the enzyme content is high, the accessibility of a particular enzyme to its preferred site is reduced, as the access is limited by other enzymes. For example, endoglucanase needs access to the amorphous region in cellulose, β-glucosidase is only efficient on oligosaccharides, and xylanase is specific to Xylan [71,72]. Limited access to these sites can lead to disorganization of the enzyme synergy and a significant drop in enzymatic hydrolysis efficiency [73].

3.6. Overview of Ex-SSF Techno-Economic Potential

Pretreatment is a key step for lignocellulosic biomass valorization, especially for applications in the biorefinery industry. Biomass pretreatment represents about 40% of the total valorization cost of second-generation bioethanol production [74]. Conventional biomass pretreatments, such as steam explosion, acid pretreatment, alkali pretreatment, microwave, ionic liquids (IL), ammonia fiber explosion (AFEX), ozonolysis, and organosolv have failed to lower the pretreatment costs to make bioethanol production not only economically viable, but also more attractive and competitive compared to fossil fuels or first-generation bioethanol [74–76]. The Ex-SSF approach provides major advantages compared to existing technologies. As evidenced in this study with black spruce and corn stover, Ex-SSF pretreatment led to interesting sugar recovery yields, similar to the most efficient conventional

methods. However, the competitive advantage of the Ex-SSF pretreatment lies in (i) the important reduction in the cost of the pretreatment, (ii) the substantial secondary revenue opportunities that Ex-SSF offers, and (iii) the scalability of the pretreatment as described next.

First, Ex-SSF is a sequential pretreatment starting with extrusion and followed by semi-solid fermentation. Extrusion is a very fast pretreatment step with low energy consumption. The mini-extruder (11 mm) used in this study had a pretreatment flow rate of about 1 kg/h with energy consumption of 40 kWh/kg, but the energy consumption with regular pilot scale extruders (36 mm and higher) can easily drop to 10 kWh/kg [77], and they can process up to 200 kg/h [78]. By upscaling the process, an important economy of scale can be achieved. Additionally, Ex-SSF does not use additives, either during the extrusion step or during the fermentations. This is a key advantage over most chemical and physico-chemical pretreatments and one less expenditure. No sophisticated reactor is required, as SSF can be carried out in simple tanks. SSF can be operated in batch, fed-batch, or continuous mode in combination with the feed coming from the extruder. As for the strain, *Phanerochaete chrysosporium* is a widely available fungus that can be isolated in the environment on decaying wood [79]. It has the advantage of flourishing in less sterile conditions in SSF, contrary to bacteria in submerged fermentation (SmF). *P. chrysosporium* in SSF has a low optimum temperature (30 °C). The Ex-SSF technology also involves very little water consumption, just enough to ensure a steady flow in the barrel for extrusion and to maintain the fungal growth for SSF. Another advantage is that SSF does not require day-to-day monitoring. Only intermittent mixing is necessary to evenly distribute the fungal colonization and avoid dead zones. Regarding secondary revenues opportunities, several options are available. For example, according to the results, Ex-SSF produced a relatively important amount of manganese peroxide (MnP). MnP could be extracted (eventually purified) and used for diverse purposes such as bioextrusion or commercialization. Furthermore, the residual biomass after enzymatic hydrolysis could be used as an input to produce composite materials with the extruder for the construction and packaging industries [80,81]. So, the extruder in the Ex-SSF approach has a double function: pretreatment and as a reactor to make materials. Composite materials are an important field of materials science, and demand for biosourced materials is growing [82]. Finally, extrusion and semi-solid fermentation are both scalable technologies, which is not always the case with conventional technologies. Today, several different sizes of extruder exist on the market, as well as vessels larger than 2000 L for SSF.

Taking everything into account, Ex-SSF is a promising approach for the pilot- or industrial-scale valorization of lignocellulosic biomass, especially black spruce and corn stover as presented here. However, a complete techno-economic analysis is required to confirm the viability of the Ex-SSF technology, for example, in a specific context such as biomass valorization in Quebec (Canada). This will be the focus of a future investigation.

4. Conclusions

The novel pretreatment approach developed in this study displayed interesting results and potential cost-effective advantages compared to existing pretreatment approaches. The manganese peroxidase (MnP) mainly responsible for the delignification process reached up to 13.8 U/L for Ex-SSF pretreated black spruce (BSE) and 32.0 U/L for Ex-SSF pretreated corn stover (CSE). This resulted in biodelignification rates of 59.1% and 65.4%, respectively. The extrusion step contributed 25.3% and 27.4%, respectively. High levels of lignin peroxide (LiP) activity were recorded in the raw BS and CS compared to their extruded biomasses. However, less than 17% biodelignification was recorded for the raw biomasses. The Ex-SSF pretreatment increased the cellulose crystallinity index (CrI) by 13% for BSE and 4% for CSE.

However, enzymatic digestibility of the Ex-SSF pretreated biomass with 0.25 mL/g enzyme led to 7.6 mg/L of sugar recovery for black spruce, which was 2.3 times the raw biomass yield. As for Ex-SSF pretreated biomass, sugar recovery of up to 17.0 mg/L was recorded, which was 44% higher than the sugar concentration in the raw corn stover. Increasing the enzyme load from 0.25 mL/g to 0.50 mg/L and 0.75 mg/L did not improve the results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/waste3030021/s1, Table S1: Delignification model results for Ex-SSF pretreated black spruce chips. Table S2: Delignification model results for Ex-SSF pretreated corn stover.

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