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Optimization of Solid-State Fermentation of Switchgrass Using White-Rot Fungi for Biofuel Production

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Abstract: Biological delignification using white-rot fungi is a possible approach in the pretreatment of lignocellulosic biomass. Despite the considerable promise of this low-input, environmentally-friendly pretreatment strategy, its large-scale application is still limited. Therefore, understanding the best combination of factors which affect biological pretreatment and its impact on enzymatic hydrolysis is essential for its commercialization. The present study was conducted to evaluate the impact of fungal pretreatment on the enzymatic digestibility of switchgrass under solid-state fermentation (SSF) using *Phanerochaete chrysosporium* (PC), *Trametes versicolor* 52J (Tv 52J), and a mutant strain of *Trametes versicolor* that is cellobiose dehydrogenase-deficient (Tv m4D). Response surface methodology and analysis of variance (ANOVA) were employed to ascertain the optimum pretreatment conditions and the effects of pretreatment factors on delignification, cellulose loss, and total available carbohydrate (TAC). Pretreatment with Tv m4D gave the highest TAC (73.4%), while the highest delignification (23.6%) was observed in the PC-treated sample. Fermentation temperature significantly affected the response variables for the wild-type fungal strains, while fermentation time was the main significant factor for Tv m4D. The result of enzymatic hydrolysis with fungus-treated switchgrass at optimum pretreatment conditions showed that pretreatment with the white-rot fungi enhanced enzymatic digestibility with wild-type *T. versicolor* (52J)-treated switchgrass, yielding approximately 64.9% and 74% more total reducing sugar before and after densification, respectively, than the untreated switchgrass sample. Pretreatment using PC and Tv 52J at low severity positively contributed to enzymatic digestibility but resulted in switchgrass pellets with low unit density and tensile strength compared to the pellets from the untreated switchgrass.

Keywords: fungal pretreatment; enzymatic digestibility; delignification; white rot fungi; cellulose loss

Citation: Onu Olughu, O.; Tabil, L.G.; Dumonceaux, T.; Mupondwa, E.; Cree, D. Optimization of Solid-State Fermentation of Switchgrass Using White-Rot Fungi for Biofuel Production. *Fuels* **2022**, *3*, 730–752. https://doi.org/10.3390/fuels3040043

Academic Editors: Gaetano Zuccaro and Neha Arora

Received: 3 October 2022
Accepted: 11 November 2022
Published: 6 December 2022

1. Introduction

The potential of dedicated energy crops as suitable feedstock for producing cellulosic ethanol has attracted increasing attention because of their high yield, low costs, decreased environmental impacts, and the lack of competition for fertile lands between energy crops and food crops [1]. Out of the many energy crops which have been studied, Wright [2] recommended further development of switchgrass as a high-potential dedicated energy crop, which has led to extensive research on its utilization and enhancement [3,4]. This development has made switchgrass a promising feedstock for cellulosic ethanol production. However, the enzymatic digestibility of lignocellulosic materials to produce sugars that can be fermented into ethanol and other platform chemicals is generally low. Studies have shown that only about 20% of the theoretical maximum yield of lignocellulose to fermentable sugar can be obtained via enzymatic hydrolysis without a pretreatment step [5,6]. The structural integrity of lignocellulose, mainly due to the presence of lignin, limits

enzyme accessibility and reduces the rate and yield of hydrolysis [7]. Other limiting factors of efficient enzymatic hydrolysis include the crystallinity of cellulose, accessible surface area, degree of cellulose polymerization, and degree of acetylation of hemicelluloses [8]. The need to overcome these constraints has made the pretreatment of lignocellulosic biomass a critical step in biofuel production. Many different biomass pretreatment options have been proposed by many investigators with promising results regarding the improvement of the enzymatic digestibility of lignocellulosic biomass [9]. Despite these promising results, the cost and environmental impacts associated with these pretreatment processes impinge on their commercial applications. Consequently, alternative strategies for more cost-effective and environmentally benign biomass pretreatment are pivotal to market-competitive cellulosic biofuel and bioproducts.

The use of microorganisms with an intrinsic capacity for delignifying cellulosic biomass as a pretreatment option is considered to be energy-saving and capable of mitigating the economic and environmental burden associated with current pretreatment strategies [10]. However, the long pretreatment times, fermentable sugar loss, inability of the microbes to selectively degrade lignin, and space requirements for large scale pretreatment associated with microbial pretreatment have put major limitations on its commercialization. Many species of wood-rotting fungi have been employed in deconstructing lignocellulose for the purpose of enhancing sugar yields during enzymatic hydrolysis. Thus, identifying organisms that selectively degrade lignin has been the focus of recent research initiatives. Among the groups of wood-rot fungi, white rot fungi are the most widely studied because they possess an enzymatic system capable of effectively degrading lignin [11]. For example, several studies have investigated the physicochemical changes in the cellulosic biomass and yield of enzymatic hydrolysis after pretreatment with white rot fungi, including *Ceriporiopsis subvermispora*, *Trametes versicolor*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium* [12–14]. Wan and Li [15] evaluated the effectiveness of fungal pretreatment by *Ceriporiopsis subvermispora* on corn stover, switchgrass, and hardwood. Glucose yields of 56.50%, 37.15%, and 24.21%, respectively, were reported, which were a two- to three-fold increase over those of the untreated materials after an 18-d pretreatment. A further 10–30% increase in glucose yields was also observed when pretreatment time was extended to 35 d. Variations in biomass feedstock, fungal species, and fungal treatment conditions greatly influence the efficiency of this pretreatment method. The essential material and process parameters influencing microbial pretreatment include the nature and composition of biomass feedstock, fungal fermentation (culture) method, type of microorganism, incubation temperature, pH, incubation time, inoculum concentration, moisture content, and aeration rate [10]. The effects of these factors on fungal pretreatment outcomes have been extensively studied. Shi et al. [16] studied microbial pretreatment of cotton stalks using *P. chrysosporium* and found that the delignification at a higher moisture content of the culture (75% and 80%) was more than that observed at a lower moisture content (65%). Similarly, the highest ligninase activity, at 70% moisture content, was reported during a study on the solid state fermentation (SSF) of corn cobs, with a moisture content ranging from 40% to 90% using *P. chrysosporium* [17]. A study on the effect of fungal culturing on the degradation of wheat straw revealed that the maximum degradation rate occurred after 19 d of fermentation [18]. Optimization of solid-state fermentation of wheat straw by *Polyporus brumalis* BRFM985 showed that, at low fermentation temperature (20 °C) and short time of cultivation, the growth of fungal mycelia was slow, which resulted in poor delignification [19]. Based on the varying responses of lignocellulosic components at various incubation times with different fungal strains [20], fermentation time should be optimized for a particular fungal strain and substrate. Fungal pretreatment can be performed under SSF or submerged fermentation (SMF). In contrast to SMF, SSF refers to the growth and/or cultivation of microorganisms on moist, water-insoluble solid substrates in the absence or near-absence of free-flowing liquid. SSF is preferred for fungal cultivation because it offers similar environmental conditions to those of the natural habitats of fungi [21]. Sahuand and Pramanik [22] demonstrated that a mixed culture of *Trametes pubscens* and

Pycnoporus cinnabarinus was more efficient in delignifying cotton gin waste under SSF than in SMF. Additionally, SSF requires low energy input which could significantly reduce the production cost at an industrial scale, as compared to the SMF [23].

At the current state of technology, the large-scale application of a fungal pretreatment strategy does not seem to be cost effective [24]. However, the selection of the best material and process conditions is pivotal in attaining the optimum efficiency in a fungal pretreatment strategy as well as a reduction in the overall cost of pretreatment for biofuel production. In addition to the cost of pretreatment, the cost of feedstock transportation to the biorefinery significantly impacts the overall cost of producing cellulosic ethanol [25]. The high cost of biomass transportation and storage is associated with the low bulk density of the biomass in its original form [26]. Pelletization has been suggested as a strategy for improving the bulk density of biomass, and thus minimizing the cost of transporting biomass over long distances [27]. It has been reported that the quality of pelletized biomass from grasses is generally low [28]; therefore, pretreatment is required prior to pelletization. The pretreatment optimization goals for a cost-effective cellulosic ethanol production should include enhanced enzymatic digestibility, improved pellet quality, and reduced cost of pretreatment. Most of the studies on the optimization of fungal pretreatment focused on either enhancing enzymatic hydrolysis [19,29] or improving pellet quality [30,31]. It is crucial to assess optimum fungal pretreatment conditions for improved enzymatic saccharification and pellet quality with the view of minimizing cost. Therefore, the present study seeks to address this research gap by determining the optimum conditions for solid state fermentation of switchgrass using white-rot fungi for enhanced enzymatic digestibility and good quality switchgrass pellets using a response surface approach.

2. Materials and Methods

2.1. Statistical Experimental Design

To examine the main and interactive effects of the pretreatment factors on the response variables, the Box–Behnken design, with four factors and three levels, was employed to optimize the fungal pretreatment conditions under solid state fermentation. The selected factors were fermentation temperature (°C), fermentation time (d), inoculum concentration (mL), and hammer-mill screen size (mm), while total available carbohydrate (%), delignification (%), and cellulose loss (%) were the response variables. Previous studies have demonstrated that temperature, inoculum concentration, particle size, and their interactions significantly impact fermentation time [32,33], which influences the overall cost of fungal pretreatment [24]. Hence, these factors were considered suitable for achieving the optimization goals, namely enhanced enzymatic hydrolysis and a minimized cost of fungal pretreatment. The levels of the factors were chosen based on preliminary trials and consultation of the literature. The actual and coded factor levels of the independent variables in the fungal pretreatment are summarized in Table 1. The multifactor experiment was designed by the interaction between the independent variables, and the responses of the dependent variables to the varied conditions were modeled via response surface methodology to obtain the optimal conditions using the Design Expert version 10.0.7 software (Stat-Ease, Inc., Minneapolis, MN, USA). A regression model that was described using a quadratic polynomial equation (Equation (1)) was fitted to the data, and analysis of variance (ANOVA) was used to assess the factor effects on dependent variables.

$$y_n = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \ (n=1, 2, 3) \quad (1)$$

where y_1 = total available carbohydrate (%); y_2 = delignification (%); y_3 = cellulose loss (%); x_1 = fermentation temperature (°C); x_2 = fermentation time (d); x_3 = inoculum concentration (mL); x_4 = hammer-mill screen size (mm); β_0, β_i, β_{ii}, and β_{ij} = the regression coefficients of intercept terms, linear terms, quadratic terms, and linear interaction terms in the equation, respectively.

Table 1. Actual and coded factor values of the independent variables in the fungal pretreatment.

Code	Actual Value			
z_j	**Fermentation Temperature, x_1 (°C)**	**Fermentation Temperature, x_2 (d)**	**Inoculum Concentration, x_3 (mL)**	**Hammer-Mill Screen Size, x_4 (mm)**
1	34	35	15	6.4
0	28	28	10	3.2
−1	22	21	5	1.6

2.2. Fungal Strains and Feedstock Preparation

Wild-type strains of white-rot fungi *Phanerochaete chrysosporium* (PC) Burdsall (ATCC 24725) and *Trametes versicolor* 52J (Tv 52J) (ATCC 2086), and cellobiose dehydrogenase-deficient strain (mutant) of the basidiomycete *Trametes versicolor* (Tv m4D) [34] used in this study were supplied by Agriculture and Agri-Food Canada, Saskatoon Research and Development Centre, Canada. The fungal strains were preserved as glycerol stocks at −80 °C and grown on Difco malt extract agar (MEA) (Benton Dickenson, Sparks, MD, USA). As substrate, switchgrass (*Panicum virgatum* L.) of the variety "Cave-in-rock" was obtained from a farm in the Nappan area (45.77° N, 64.24° W) of Nova Scotia, Canada. Switchgrass samples were ground using a hammer mill to three different screen sizes (6.4, 3.2, and 1.6 mm). The method described in our previous study [30] was used to prepare inoculation cultures for solid state fermentation.

2.3. Fungal Pretreatment

To prevent the growth of microorganisms other than the organism of interest, the biomass feedstock was subjected to heat treatment using an autoclave at 121 °C for about 20 min before inoculation. Approximately 20 g of the sterilized switchgrass was inoculated at different inoculation volumes (5-, 10-, and 15-mL) in a plastic vented bag, which provided room for gas exchange. The moisture content of the cultures was adjusted to 80% (w.b.) using sterile water. The inoculated substrates were incubated at 22, 28, and 34 °C for 21, 28, and 35 d. Ambient humidity was stabilized by placing a tray of water inside the incubator. The fungal-pretreated switchgrass samples were prepared for compositional analysis following NREL protocol [35].

2.4. Compositional Analysis

The fungal-treated and untreated switchgrass was hydrolyzed by acid hydrolysis [36]. The monomeric sugar content of the hydrolysates was detected using an Agilent HPLC (Agilent 1100, Agilent Technologies, Santa Clara, CA, USA). An Aminex HPX-87P. 300 mm × 7.8 mm column with a refractive index detector at temperature of 85 °C and flowrate of 0.6 mL/min was used in the HPLC analysis. The amounts of cellulose and hemicellulose present were computed from the concentration of the individual sugar monomers detected using the calibration curves developed from the calibration standards (D (+) glucose, D (+) xylose, and other C5 sugars). Glucose content roughly approximates cellulose content, while hemicellulose was calculated as the sum of all of the 5-carbon sugars present in the sample. The retentate was used to determine the quantity of acid-insoluble lignin by gravimetric analysis, while the total acid-soluble lignin (ASL) was measured using the absorbance of the acid hydrolysate determined with an ultraviolet visible light spectrophotometer (UV mini-1240, Shimadzu Corp., Kyoto, Japan) at a wavelength of 240 nm. ASL was quantified using Equation (2). Delignification, percentage of cellulose loss, and selectivity value were evaluated using Equations (3)–(5).

$$\text{Soluble lignin content} = \frac{A_{240} * 87\ \text{mL} * \text{dilution factor}}{\varepsilon * \text{dried sample mass} * \text{spec pathlength}} * 100\% \quad (2)$$

where ε is the feedstock-dependent absorptivity constant

$$\text{delignification} = \frac{\text{Li}_{\text{initial}} - \text{Li}_{\text{final}}}{\text{Li}_{\text{initial}}} \times 100 \quad (3)$$

$$\%\ \text{cellulose loss} = \frac{\text{Cel}_{\text{initial}} - \text{Cel}_{\text{final}}}{\text{Cel}_{\text{initial}}} \times 100 \quad (4)$$

$$\text{Selectivity value (SV)} = \frac{\text{delignification}}{\%\ \text{cellulose loss}} \quad (5)$$

where $\text{Li}_{\text{initial}}$ = percentage of total lignin content in raw material; Li_{final} = percentage of total lignin content in fungal-treated material; $\text{Cel}_{\text{initial}}$ = percentage of cellulose content in raw material; $\text{Cel}_{\text{final}}$ = percentage of cellulose content in fungal-treated material.

2.5. Enzymatic Hydrolysis

To evaluate the potential of the fungal pretreatment of switchgrass for enhanced cellulose and hemicellulose accessibility, enzymatic hydrolysis of the carbohydrate fractions was investigated using switchgrass samples pretreated at optimum fungal pretreatment conditions. The enzymatic digestibilities of the untreated and fungal-treated switchgrass samples was determined before and after densification using a single pelleting unit mounted on an Instron tester (Model No. 3366, Instron Corp., Norwood, MA, USA), following the NREL standard protocol [37]. A total of 14 mg of the biomass slurry was transferred to a 2.0 mL glass HPLC vial. A total of 42 µL of 1.0 M sodium citrate buffer at pH 5.0 and 5.6 µL of a 5.0% sodium azide solution were added to the vial. A total of 8.0 µL of cellulase (Sigma–Aldrich, St. Louis, MO, USA) and 21.7 µL of β-glucosidase (Sigma–Aldrich, St. Louis, MO, USA) were added after bringing the volume of the mixture to 1.4 mL with distilled water. The protein concentration and volume of the enzymes used were determined using Pierce BCA protein assay and Equation (6), respectively. The enzymatic hydrolysis experiment was performed in three replicates using a water bath with a shaker at 50 °C and an agitation rate of 150 rpm for 72 h. The hydrolysate was passed through a 0.2 µm syringe filter to further remove any insoluble material. The Dinitrosalicyclic (DNS) method described by Miller [38] was used to measure the total reducing sugar yield. A total of 1 mL of the DNSA reagent was added to 1 mL of the filtrate in a test tube. The test tubes were incubated in a boiling water bath for 5 min. The absorbance, which is a function of the concentration of the reducing sugar in the solution, was quantified spectrophotometrically at a wavelength of 540 nm. The reducing sugar yield was obtained from the standard calibration curve developed with standard glucose solutions. Percentage digestion was calculated using Equation (7)

$$\text{Enzyme volume} = \frac{1.0\ \text{mL}}{\text{X mg protein}} \times \frac{20.0\ \text{mg protein}}{1.0\ \text{g glucan}} \times \text{g glucan} \quad (6)$$

where X = mg protein in 1.0 mL of enzyme sample; g = g of glucan in biomass sample.

$$\%\ \text{digestion} = \frac{\text{grams cellulose digested}}{\text{grams cellulose added}} \times 100 \quad (7)$$

2.6. Pelletization, Raw Materials and Pellet Characterization

Switchgrass samples pretreated under optimum fungal pretreatment conditions were pelleted using a single pelleting unit (SPU), and pellet properties such as pellet tensile strength, unit, and relaxed density (density immediately after pelleting and density after 14 d of pelleting) were evaluated as described previously [31]. Synchrotron-based computed tomography (SR-CT) was employed in imaging the internal void structure of the pellets. All SR-CT scans were acquired at the biomedical imaging and therapy beamline-bending magnet (BMIT-BM beamline) at the Canadian Light Source (Saskatoon, SK, Canada). Data were collected under the following instrument conditions: beam energy: 20 keV; sample-

detector distance: 20 cm; exposure time: 950 ms; number of projections: 1800; pixel dimensions: 8.881 mm; size of imaged area: 16 × 4.5 mm. Image J and NRecon image processing software packages were used for the adjustment and reconstruction of CT projection images, while image visualization and porosity determination were performed using Avizo imaging software.

The ultimate analysis of the untreated and fungal-treated switchgrass samples were performed using an Elemental Analyzer (Vario EL III Elementar, Analysensysteme GmbH, Hanau, Germany). Each test was replicated three times. The percentage of oxygen content in the sample was obtained by subtracting the sum of the percentages of carbon, hydrogen, nitrogen, sulfur, and ash content from 100%. The crude protein content of the untreated switchgrass sample was determined using the Kjeldahl method, according to the AOAC 981.1 standard [39]. The total ash content was determined using the NREL standard method [40], where 0.5–2 g of sample was heated for 24 ± 6 h at 575 ± 20 °C in a preheated muffle furnace (model no. F-A1730; Thermolyne, Dubuque, IA, USA). Mineral compositions of the ashes were characterized using inductively coupled plasma-mass spectrometry (ICP−MS) (Sciex Elan 5000, PerkinElmer Inc., Waltham, MA, USA).

3. Results and Discussion

3.1. Characterization of the Raw Material

The mean chemical composition of the untreated switchgrass was determined to be 51.4% of the total available carbohydrates (36.3 ± 1.6% cellulose and 15.1 ± 0.3% hemicellulose), 26.8 ± 0.2% of the total lignin, 2.2 ± 0.03% crude protein, and 3.2 ± 0.1% ash content. The hemicellulose reported in this study comprised only xylose, while glucose is inferred to represent cellulose. Arabinose, mannose, and galactose were ignored because their amounts were below measurement sensitivities. In comparison with the composition of different cultivars of switchgrass analyzed by other investigators [41,42], the raw material used in this study had an approximately equal percentage of glucose content, less hemicellulose, slightly varying ash, and higher lignin. However, the lignin content agreed with the findings of Hu and Wen [43] and Karunanithy and Muthukumarappan [44]. Hemicellulose content was considerably lower than values reported in the above-named studies, probably because the other C5 sugars (arabinose, galactose, and mannose) were not quantified. On the other hand, the relatively high lignin observed in the raw material could constitute a barrier to efficient sugar recovery during enzymatic hydrolysis.

3.2. Fungal Growth

The visual examination of all twenty-nine experimental runs conducted in this study showed that the exposed surfaces of the substrates were almost covered with the fungal mycelia after the specified fermentation times, which shows that the fungal strains were able to grow on switchgrass. This observation was true for all three fungal strains used in this research. However, as noted in previous studies [45,46], the mutant strain of *T. versicolor* (Tv m4D) had minimal growth on switchgrass as compared to the wild-type fungal strains, especially PC, and was more conspicuous after 21 d of fermentation. The slow growth rate of Tv m4D is believed to be associated with the reduced capacity for cellulose catabolism due to lack of cellobiose dehydrogenase (CDH) in this strain [34]. The deficiency in functional CDH in Tv m4D is intended to preserve the cellulose fraction to have more available carbohydrate for cellulosic ethanol production.

3.3. Fungal Pretreatment

The percentage totals of available carbohydrate, cellulose loss, and delignification and selectivity value of switchgrass pretreated with PC, Tv 52J, and Tv m4D under solid state fermentation are shown in Table S1. In comparison to the untreated sample, the highest percentage totals of available carbohydrate and delignification were observed to be 66.7% and 23.6%, 67.6% and 21.7%, and 73.4% and 22.4% in the *P. chrysosporium*-, *T. versicolor* 52J-, and *T. versicolor* m4D-treated switchgrass samples, respectively. The degradation of

the switchgrass samples was dependent on fungal strains. This is seen in the selectivity values of the fungal strains as illustrated in Table S1. Negative selectivity values signify an apparent increase in the percentage of lignin or cellulose content with respect to the raw material. Glucose from the fungal cell wall possibly contributed to the apparent increase in total available carbohydrate in the fungal-treated materials. For *P. chrysosporium* treated samples, the highest selectivity value of 15.4 and lowest cellulose loss of 0.8% were recorded after 21 d of fermentation (experimental run #12), while the lowest selectivity value of −0.1 and 39.4% loss in cellulose were obtained after 35 d of fermentation. This implies that most of the lignin degradation occurred within the first three weeks of fermentation and a longer fermentation time results in a high rate of cellulose loss. Although the highest delignification was obtained in experimental run #5, it also occurred after 21 d of fermentation but at a lower fermentation temperature and hammer-mill screen size. In the same vein, *T. versicolor* 52J and the mutant strain (Tv m4D) recorded high and low selectivity values of 42.07 and 0.05, and 38.67 and −0.16, respectively. Unlike the PC, the *T. versicolor* strains exhibited a high selectivity value after 28 d of fermentation. Similarly, a two-fold increase in the selectivity value of PC as compared to *Trametes* was reported for the solid-state fermentation of oil palm empty fruit bunch [47]. This suggests that lignin degradation commenced faster in the PC-treated switchgrass samples than in the *T. versicolor* strains, which implies that PC possesses a more efficient enzymatic system which is capable of deconstructing lignocellulosic materials. This observation provides more explanation for the rapid growth of PC mycelia on the substrates within the first 21 d of fermentation as mentioned earlier. Intense delignification during the early stage of fermentation, exhibited by PC relative to the other fungal strains, agrees with the report of Ganesh Kumar et al. [13], where increased lignin degradation in *Achras zapota* lignocellulose was recorded in the first 7 d of solid-state fermentation. Similarly, the low cellulose degradation within 21 d of fermentation noted in this work aligns with the study on solid state fermentation of radiata pine using *T. versicolor* [48]. According to that study, pretreatment with *T. versicolor* led to 5% cellulose loss in 21 d and about 40% cellulose loss after 35 d of fermentation. This is consistent with previous work on the pretreatment of canola straw using Tv 52J, which demonstrated a significant reduction in the level of glucose in canola straw after 12 weeks of fermentation [45].

Furthermore, the range of values between the highest and lowest selectivity value obtained for the three fungal strains displayed in Table S1 showed that PC had the lowest range (15.03). High and low selectivity values indicate a preference for lignin and cellulose degradation, respectively. A selectivity value close to 1 shows concurrent degradation of the carbohydrates and lignin in an approximately equivalent amount. Hence, it can be inferred from the selectivity value listed in Table S1 that PC simultaneously degraded holocellulose and lignin while the *Trametes* strains showed greater selectivity for lignin. These findings corroborate previous studies on the non-selective nature of PC [16].

3.4. Effect of Pretreatment on Delignification

The analysis of variance (ANOVA) for the main and interaction effects of fermentation temperature, time, inoculum concentration, and hammer-mill screen size on fungal delignification is presented in Table 2. The results indicate that, for all three fungal strains, the best-fitting models for all of the response variables were highly significant ($p < 0.01$), while the lack of fit for all response variables were insignificant. The low probability values of the multivariate regression models show the existence of correlation between the pretreatment factors investigated and fungal delignification for all three fungal strains. For pretreatment with PC, the main effects of fermentation temperature, time, and hammer-mill screen size were statistically significant ($p < 0.05$) in terms of lignin degradation. A significant effect of fermentation time on delignification was also observed in the solid state cultivation of cotton stalk using PC [16]. Similarly, fermentation temperature was the main significant effect for lignin degradation for the solid-state fermentation with Tv 52J, while the main effect of time and inoculum concentration were statistically significant at $p < 0.01$ and

$p < 0.1$, respectively, for lignin degradation with Tv m4D. The significant effect of inoculum concentration on delignification noted in the mutant strain (Tv m4D) suggests that, at any given pretreatment time, the addition of inoculum to the substrate will likely alter the rate of lignin degradation. Unlike the wild-type strains, the effect of interaction of inoculum concentration and fermentation time was statistically insignificant for delignification with the mutant strain, which implies that the effect of time on delignification is independent of the variation in inoculum concentration. Together, these observations further highlight the slow growth of the Tv m4D on the substrate relative to the wild-type strain, which is as expected. The Tv m4D is a genetically modified strain with a feature that limits its growth on the substrate with the purpose of reducing cellulose degradation [34].

The developed quadratic models for delignification for all three fungal strains, in terms of coded variables shown in Equations (8)–(10), underline the relative impact of the independent variables based on their coefficients. Positive coefficients signify a linear increase in delignification while negative coefficients signify a linear decrease in delignification. With respect to the magnitude of coefficients, fermentation temperature is the most important linear factor for delignification using PC and Tv 52J. However, temperature had a positive influence on delignification for PC and a negative influence on delignification for Tv 52J. On the other hand, fermentation time had the highest and a positive impact on delignification for Tv m4D treatment, whereas a negative effect of time on delignification was observed for PC. However, for PC, the quadratic term of fermentation time contributed positively to delignification. A decreasing trend in the delignification rate with increasing fermentation time was reported by [49] when rice straw was treated with PC under solid-state fermentation for 10–40 d.

Quadratic models for delignification:

P. chrysosporium:

$$\text{Delignification} = -22.4 + 8.3X_1 - 6.9X_2 + 6.9X_4 + 11.8X_1^2 + 16.7X_2^2 + 12.7X_3^2 + 16.0X_4^2 \quad (8)$$

T. versicolor 52J:

$$\text{Delignification} = 5.6 - 5.6X_1 + 19.6X_1X_4 + 13.6X_2X_3 - 8.3X_1^2 - 10.7X_4^2 \quad (9)$$

T. versicolor m4D:

$$\text{Delignification} = 7.8 + 6.7X_2 + 3.8X_3 + 5.5X_4^2 \quad (10)$$

As observed in Table 3, an interactive effect of factors on delignification was only noted in *T. versicolor* 52J. The ANOVA results showed that the interactive effects of fermentation temperature and hammer-mill screen size, and fermentation time and inoculum concentration, on delignification were highly significant ($p < 0.01$), indicating that these interactions had a huge impact on delignification. Contour plots displayed in Figure 1 further elucidate the effect of the factor interaction on delignification for Tv 52J. A linear contour curve indicates that the interactive effect of the factors on the response is of little importance, whereas, if the contour curve has a considerable curvature, it implies that the effect of the interaction of factors on response is significant and paramount. Figure 1a illustrates how the interaction of fermentation temperature with hammer-mill screen size affects delignification with time and inoculum concentration being constant at 28 d and 10 mL, respectively. The darker regions (red) identify higher percentages of delignification, while regions with color close to blue represent low delignification. Contour levels reveal a peak percentage delignification at a fermentation temperature of 23 °C and hammer mill size of 2.8 mm. However, the delignification in this peak region is greater than 10% (21.7%). It can be observed from the contour plot that regions of positive delignification are located at low temperatures between 22 °C and about 26.5 °C and a corresponding hammer-mill screen size of 1.6–4.0 mm. This suggests that solid-state fermentation using Tv 52J within this region resulted in a low-percentage lignin content of pretreated material relative to

the lignin content of the raw material. The range of particle sizes which supported fungal delignification reported in this study shows the importance of striking a balance in selecting substrate particle size for solid state cultivation. Zadrazil and Puniya [50] noted that a large particle size can prevent the diffusion of air, water, and metabolite intermediates into the particles, while a small particle size with low porosity reduces interparticle gas circulation. This was further confirmed by studies on the effects of substrate particle size and additional nitrogen source on the production of lignocellulolytic enzymes by *P. ostreatus* using sugarcane bagasse as substrate [51]. Therefore, for fungal pretreatment using Tv 52J, at 28 d of fermentation and a 10 mL inoculum concentration, values of temperature and hammer-mill screen size within the highlighted region (close to red) on the contour plot should be recommended for high-percentage delignification. Similarly, the contour plot of the impact of the interaction of fermentation time with temperature on delignification depicted in Figure 1b shows that lignin degradation increases with an increase in fermentation time at temperatures between 25 °C and 28 °C. This observation agrees with our previous study on Tv 52J [31], which reported a more dimensionally stable switchgrass pellet at fermentation temperatures above 25 °C and about 35 d of fermentation. Lignin degradation improves the particle binding of pellets and, consequently, their dimensional stability. White rot basidiomycetes attain a high delignification rate within an optimal temperature range of 25 and 30 °C [52]. The interactive effect of fermentation temperature and time on delignification further illustrates why the delignification peak (21.7%) was obtained at a temperature of 28 °C and 35 d of fermentation.

Table 2. Analysis of variance showing the effect of fungal pretreatment conditions on delignification.

Fungal Strain	Analysis of Variance (ANOVA)					
	Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**
P. chrysosporium	Model	5492.82	7	784.69	6.30	0.0005
	X_1	824.01	1	824.01	6.61	0.0178
	X_2	573.95	1	573.95	4.61	0.0437
	X_4	568.79	1	568.79	4.56	0.0446
	X_1^2	896.13	1	896.13	7.19	0.0140
	X_2^2	1810.57	1	1810.57	14.53	0.0010
	X_3^2	1052.01	1	1052.01	8.44	0.0085
	X_4^2	1661.83	1	1661.83	13.33	0.0015
	Lack of Fit	2388.60	17	140.51	2.46	0.1989
T. versicolor 52J	Model	3761.84	5	752.37	8.93	<0.0001
	X_1	373.14	1	373.14	4.43	0.0465
	X_1X_4	1540.75	1	1540.75	18.29	0.0003
	X_2X_3	734.56	1	734.56	8.72	0.0071
	X_2^2	470.95	1	470.95	5.59	0.0269
	X_4^2	788.78	1	788.78	9.36	0.0056
	Lack of Fit	1383.35	19	72.81	0.53	0.8487
T. versicolor m4D	Model	930.30	3	310.10	6.60	0.0019
	X_2	543.35	1	543.35	11.57	0.0023
	X_3	176.19	1	176.19	3.75	0.0641
	X_4^2	210.76	1	210.76	4.49	0.0442
	Lack of Fit	1126.15	21	53.63	4.50	0.0769

X_1 = fermentation temperature (°C); X_2 = fermentation time (d); X_3 = inoculum concentration (mL); X_4 = hammer-mill screen size (mm).

Table 3. Analysis of variance (ANOVA) showing the effect of fungal pretreatment conditions on total available carbohydrate and cellulose loss.

Fungal Strain		Total Available Carbohydrate					Cellulose Loss				
	Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	Sum of Squares	df	Mean Square	F-Value	*p*-Value
PC	Model	515.20	4	128.80	4.33	0.0089	1390.65	3	463.55	4.30	0.0142
	X_1	192.80	1	192.80	6.48	0.0178	615.62	1	615.62	5.70	0.0248
	X_1X_3	113.42	1	113.42	3.81	0.0627	361.31	1	361.31	3.35	0.0792
	X_3^2	92.81	1	92.81	3.12	0.0902	413.72	1	413.72	3.83	0.0615
	X_4^2	143.96	1	143.96	4.84	0.0378	2451.76	21	116.75	1.90	0.2833
	Lack of Fit	680.58	20	34.03	4.01	0.0933					
Tv52J	Model	361.64	3	120.55	5.19	0.0063	1753.37	3	584.46	7.26	0.0012
	X_3	197.64	1	197.64	8.51	0.0074	937.40	1	937.40	11.65	0.0022
	X_1X_3	94.09	1	94.09	4.05	0.0550	461.72	1	461.72	5.74	0.0244
	X_2^2	69.91	1	69.91	3.01	0.0951	354.25	1	354.25	4.40	0.0462
	Lack of Fit	526.85	21	25.09	1.87	0.2890	1870.86	21	89.09	2.53	0.1905
Tvm4D	Model	611.05	3	203.68	6.06	0.0030	2813.24	3	937.75	5.35	0.0055
	X_2	141.11	1	141.11	4.20	0.0511	2813.24	3	937.75	5.35	0.0055
	X_1^2	382.17	1	382.17	11.37	0.0024	1672.41	1	1672.41	9.54	0.0049
	X_3^2	143.12	1	143.12	4.26	0.0496	727.02	1	727.02	4.15	0.0524
	Lack of Fit	674.34	21	32.11	0.78	0.6942	3562.31	21	169.63	0.83	0.6624

PC = *P. chyrsosporium*; Tv52J = *T. versicolor* 52J; Tvm4D = *T. versicolor* m4D; X_1 = fermentation temperature (°C); X_2 = fermentation time (d); X_3 = inoculum concentration (mL); X_4 = hammer-mill screen size (mm).

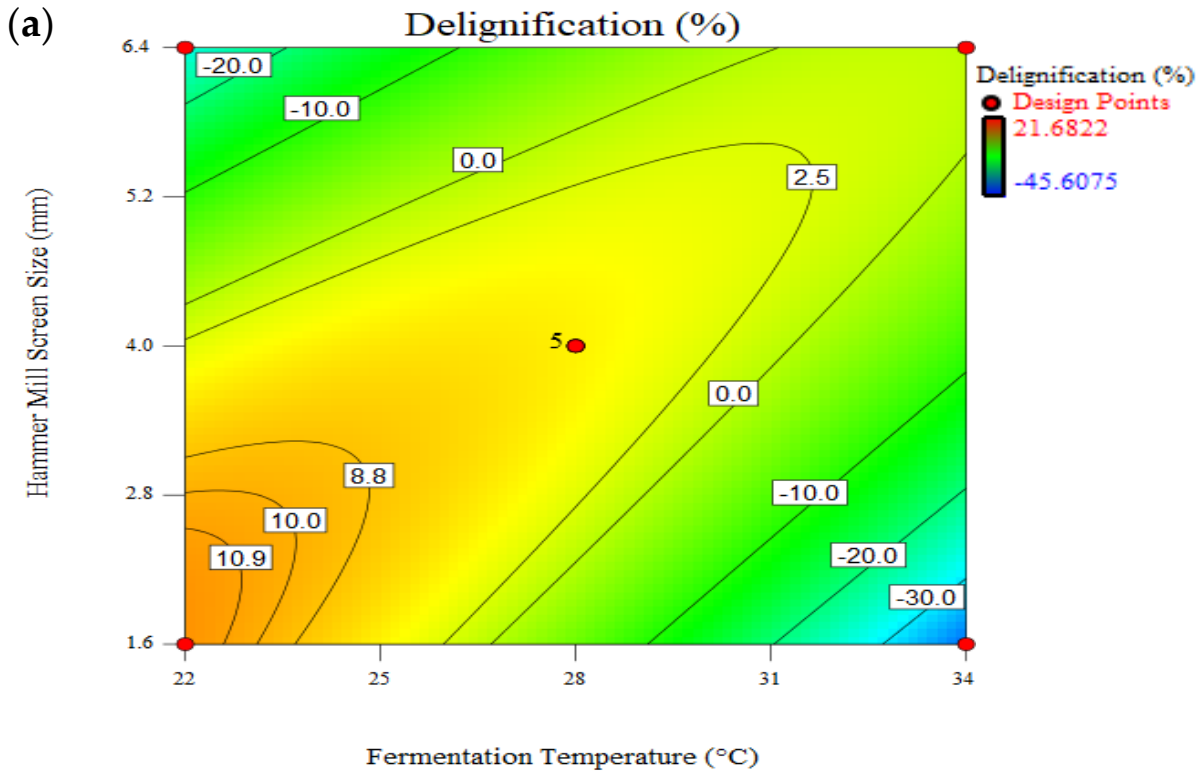

Figure 1. *Cont.*

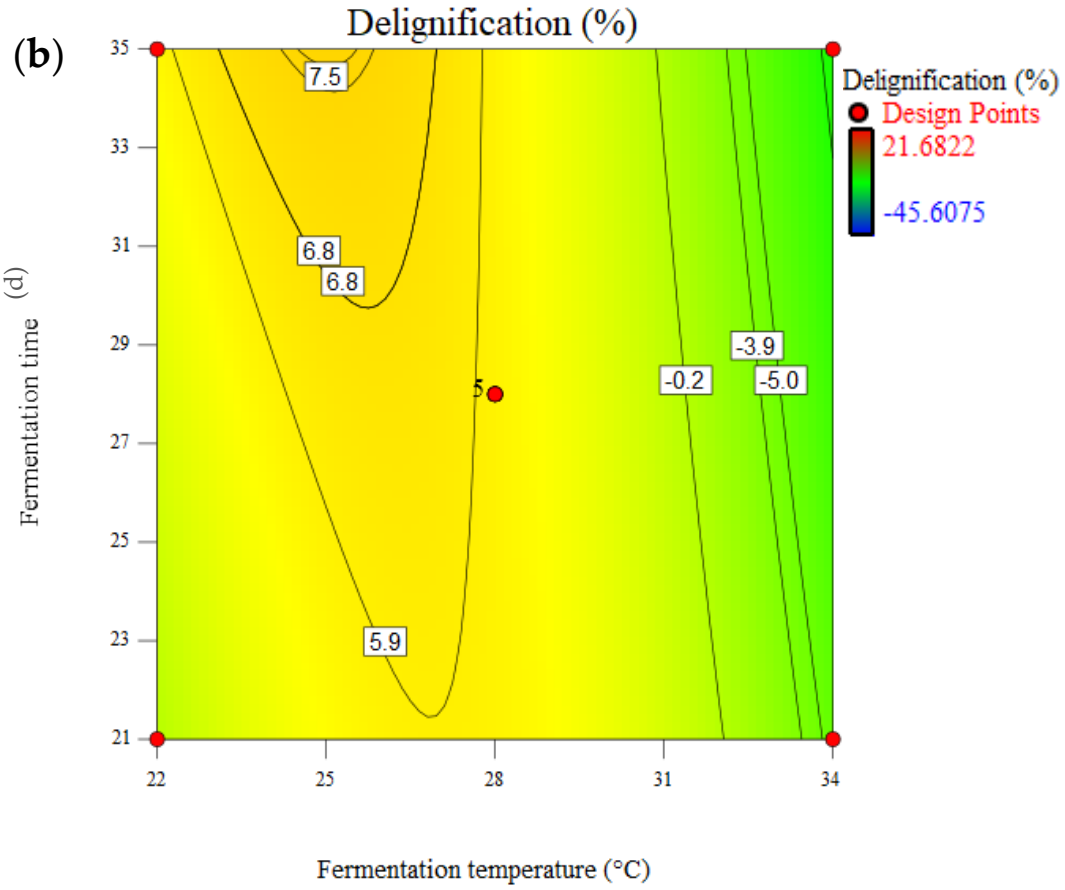

Figure 1. Effect of the factor interaction on delignification for *T. versicolor* 52J. (**a**) Fermentation temperature vs. hammer-mill screen size, (**b**) fermentation temperature vs. fermentation time.

3.5. Effect of Pretreatment on Total Available Carbohydrate (TAC) and Cellulose Loss

The TAC and percentage of cellulose loss for all three fungal strains are listed in Table S1. The mean TAC ranged between 36.8 and 66.7%, 36.1 and 67.6%, and 41.0 and 73.4% for PC, Tv 52J, and Tv m4D, respectively. In the same vein, the mean percentage of cellulose loss was between 0.8 and 39.4%, 0.3 and 45.2%, and 2.2 and 32.8% for PC, Tv 52J, and Tv m4D, respectively. A negative percentage of cellulose loss implies that the fungal-pretreated sample has a higher percentage of cellulose content than the raw material. This increase in cellulose is attributed partly to the contribution of glucose from the fungal biomass which was not accounted for. An ANOVA was performed for the TAC and percentage of cellulose loss for all three fungal strains, and the results are presented in Table 3. For TAC, the *p*-values for the multivariate regression models were less than 0.01, indicating that the models were statistically highly significant. The lack of fit was highly insignificant ($p < 0.01$), which further indicates the reliability of the models. Analysis of the response trends showed that the model could only explain the influence of fermentation temperature, inoculum concentration, and fermentation time on total available carbohydrate for PC, Tv 52J, and Tv m4D, respectively. The linear term of fermentation time and quadratic term of the hammer-mill screen size were significant ($p < 0.05$), while the quadratic term of inoculum concentration and interactive effect of temperature and inoculum concentration were sightly significant ($p < 0.1$) in pretreatment using PC. For Tv 52J, the linear term of inoculum concentration and quadratic term of time were significant at $p < 0.01$ and $p < 0.1$, respectively, whereas the quadratic term of temperature and inoculum concentration had a significant ($p < 0.05$) effect on the total available carbohydrate. The coefficients calculated by regression analysis for each of the statistically significant independent variables are presented in Equations (11)–(13).

Quadratic models for total available carbohydrate (TAC):
P. chrysosporium:

$$\text{TAC} = 48.6 + 4.0X_1 + 5.3X_1X_3 + 3.7X_3^2 + 4.6X_4^2 \quad (11)$$

T. versicolor 52J:

$$\text{TAC} = 54.3 + 4.1X_3 + 4.9X_1X_3 + 3.2X_2^2 \quad (12)$$

T. versicolor m4D:

$$\text{TAC} = 47.6 + 3.4X_2 + 7.4X_1^2 + 4.6X_3^2 \quad (13)$$

All the terms in the equations for all three fungal strains had positive coefficients, which means they positively impacted the available carbohydrate. Figure 2 displays contour plots on the response of the total available carbohydrate to the interaction of fermentation temperature with inoculum concentration. From Figure 2a, it can be observed that an increase in temperature with an increase in inoculum concentration resulted in more available carbohydrate in the pretreated biomass. The peak percentage total of available carbohydrate (67.6%) was obtained at a temperature of 28 °C and inoculum concentration of 15 mL. This suggests that pretreatment with Tv 52J at temperatures well above room temperature and high inoculum concentration will give a carbohydrate-rich material. For *P. chrysosporium*, the response of TAC to the interactive effect of temperature and inoculum concentration shown in Figure 2b had a similar trend to that of Tv 52J for higher values of TAC. However, the trend is quite different for lower values of TAC, which is represented by the region marked with a blue color on the contour plot. It can be inferred from the contour plot that solid-state fermentation at temperatures below 25 °C and inoculum concentration of greater than approximately 9.5 mL with fermentation time and hammer-mill screen size at 28 d and 3.2 mm, respectively, will lead to a high percentage of cellulose loss. This observation is further elucidated in the effect of the interaction of temperature with inoculum concentration on the percent of cellulose loss presented in Figure 3. In all three fungi, it should be noted that a temperature below 28 °C favors delignification and percentage of cellulose loss, which probably indicates reduced metabolic activities in the white rot fungi at higher temperatures. This finding supports the assertion that the accumulated heat generated due to the metabolic activity of white rot fungi during solid-state fermentation inhibits fungal growth and metabolism [53]. Therefore, an efficient bioreactor for solid-state fermentation using the white rot basidiomycetes should have a means for heat dissipation.

As expected, the percentage of cellulose loss exhibited a similar response to that of TAC in terms of statistical significance of the factors studied as can be seen in Table 3. Contrary to the TAC, all terms in Equations (14)–(16) had negative coefficients, which means the effect of the variables on cellulose loss was opposite to that of the total available carbohydrate. This observation is vividly demonstrated in the contour plots of the interaction effect of temperature with inoculum concentration on the percentage of cellulose loss in Tv 52J- and PC-treated samples depicted in Figure 3a,b, respectively. The interactive effect of these factors on cellulose loss is the reverse of the effect on total available carbohydrate.

Quadratic models for cellulose loss:
P. chrysosporium:

$$\text{Cellulose loss} = 12.6 - 7.2X_1 - 9.5X_1X_3 - 7.7X_4^2 \quad (14)$$

T. versicolor 52J:

$$\text{Cellulose loss} = 6.1 - 8.8X_3 - 10.7X_1X_3 - 7.1X_2^2 \quad (15)$$

T. versicolor m4D:

$$\text{Cellulose loss} = 18.9 - 7.5X_2 - 15.6X_1^2 - 10.3X_3^2 \quad (16)$$

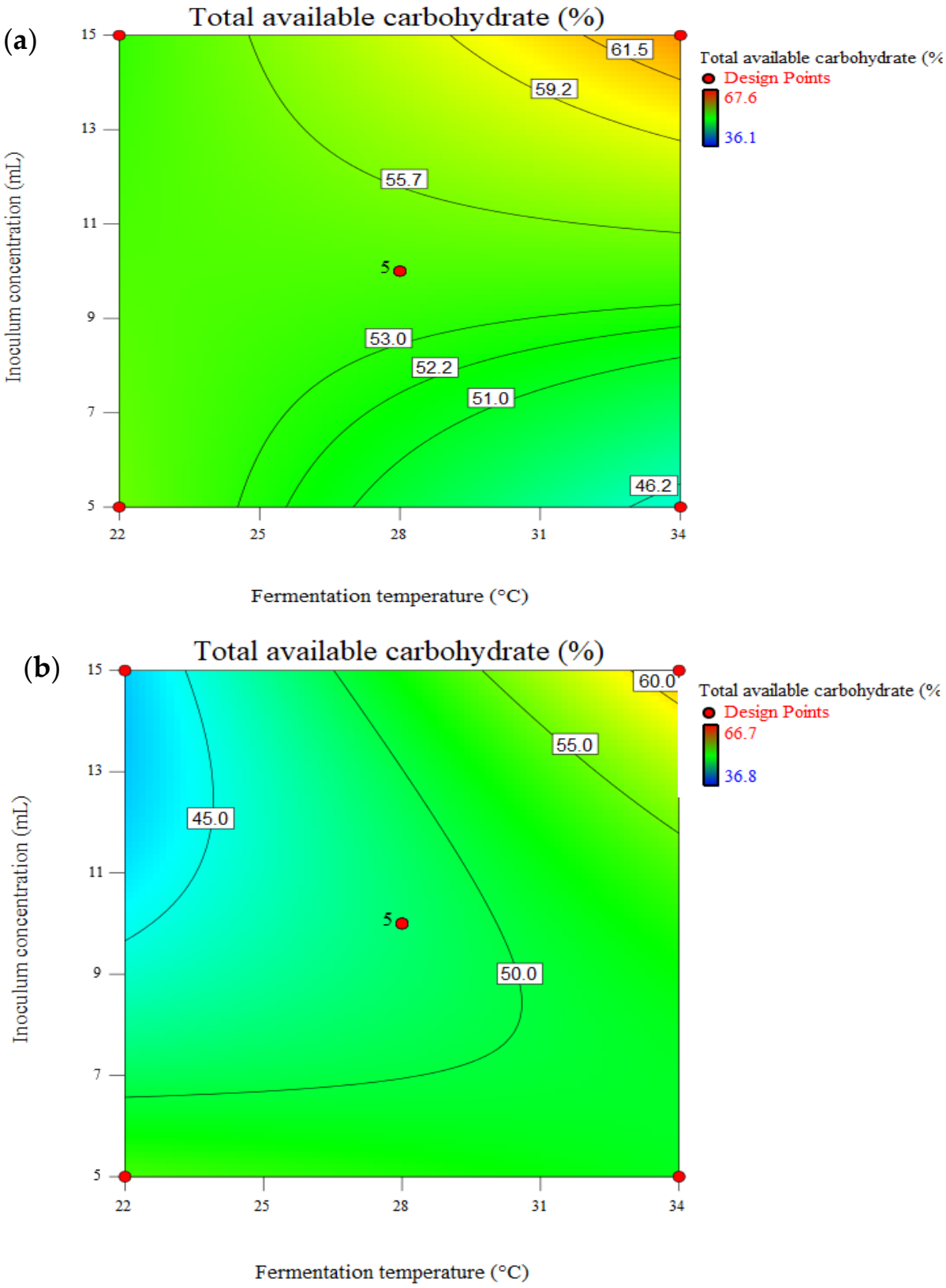

Figure 2. Interactive effect of fermentation temperature and inoculum concentration on total available carbohydrate for (**a**) *T. versicolor* 52J and (**b**) *P. chrysosporium*.

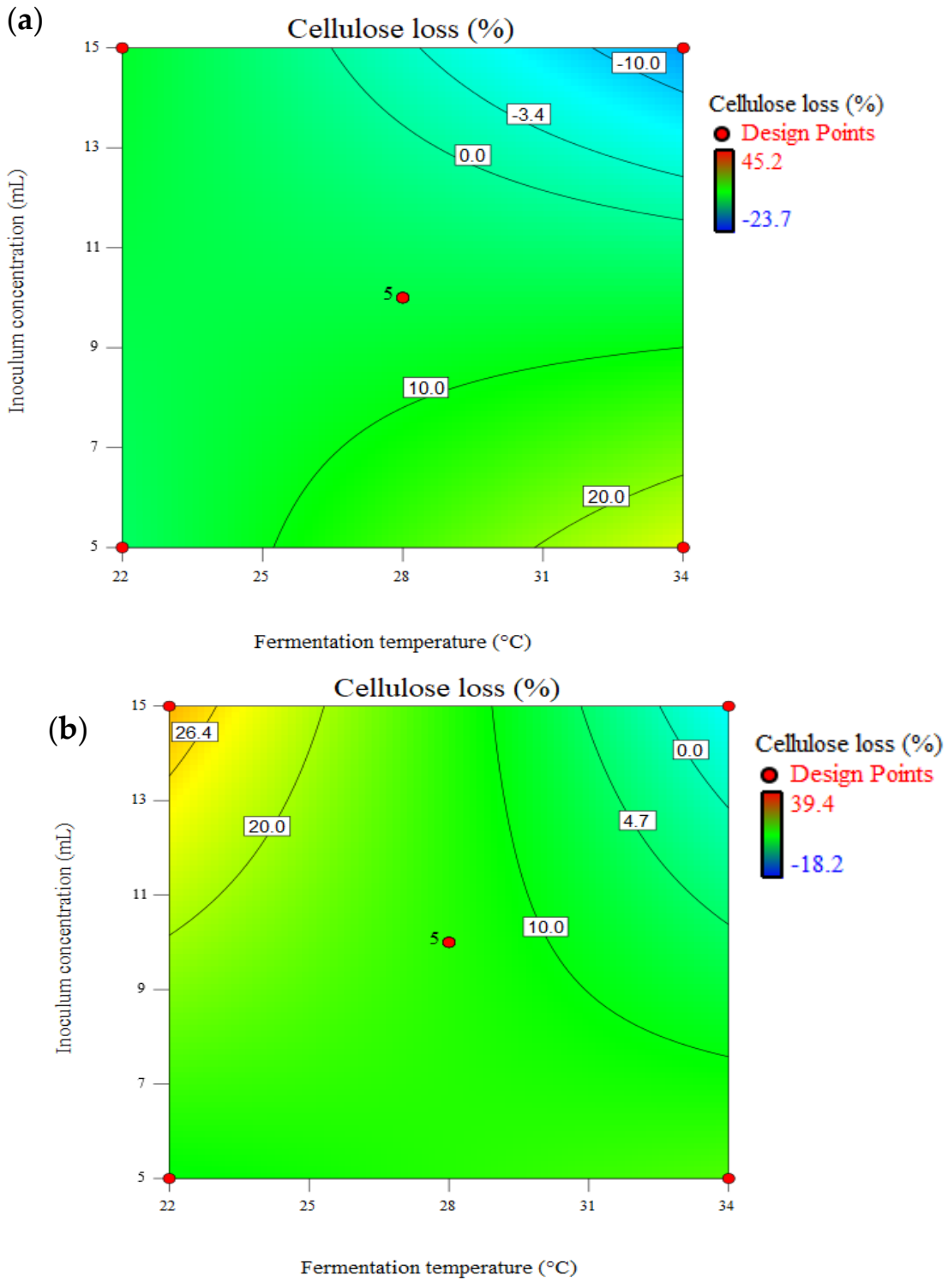

Figure 3. Interactive effect of fermentation temperature and inoculum concentration on cellulose loss for (**a**) *T. versicolor* 52J and (**b**) *P. chrysosporium*.

3.6. Optimization and Enzymatic Hydrolysis

Based on the models, numerical optimization was carried out in Design Expert for all three fungal strains. The optimization criteria for the response variables were to maximize the percentage of total available carbohydrate and delignification, and to minimize the percentage of cellulose loss. The pretreatment factors were kept in range between lower and upper limits. One hundred optimum solutions were found, and the two solutions

that best suited the optimization goals for all three fungal strains are shown in Table 4. Solution #1 was selected as the best optimum condition due to a high percentage of total available carbohydrate and delignification which is believed to favor the output of enzymatic hydrolysis as reported in previous work [54]. Yu et al. [55] investigated the enzymatic hydrolysis of rice hull after biological pretreatment and observed that more delignified material at longer fermentation times gave a higher percent total of soluble sugar after enzymatic hydrolysis. On the other hand, solution #2 was chosen as the best condition in terms of pretreatment severity, which impacts the cost effectiveness of a pretreatment strategy. Low pretreatment severity (low temperature and inoculum concentration, short fermentation time, and high hammer-mill screen size) is expected to reduce the overall cost of pretreatment, which accounts for approximately 20% of the total cost of cellulosic ethanol production [56]; therefore, this constitutes a major bottleneck in the commercial application of the cellulosic biorefinery concept. Solid-state fermentation of switchgrass at two different optimum conditions (solutions #1 and #2) was conducted for *P. chrysosporium* and *T. versicolor* 52J. The pretreated samples and their pellets were subjected to enzymatic hydrolysis as described in Section 2.6. Figure 4 depicts the results of total reducing sugar and percent of digestion obtained after enzymatic hydrolysis of the fungal-pretreated switchgrass samples. In all of the cases which were studied, fungal pretreatment resulted in a higher total reducing sugar and improved the efficiency of enzymatic hydrolysis relative to the raw material, which gave a mean total reducing sugar of 41.81 $\pm$ 0.9 mg g^{-1} and percentage of digestion of 11.52%. Tv 52J under solution #2 presented the highest total reducing sugar (TRS) yield (68.96 mg g^{-1}) and percentage of digestion (19%) followed by PC under solution #1 (64.09 mg g^{-1} and 17.65%), which is about a 64.9% and 53.3% increase, respectively, as compared to the TRS yield of the untreated sample. A similar trend was exhibited after the solid-state fermentation of switchgrass with the fungus *Pycnoporus* sp., where enzymatic digestibility increased after 18 and 36 d of fermentation as compared to 54 and 72 d [57].

Table 4. Optimum fungal pretreatment conditions for enhanced enzymatic hydrolysis.

Fungal Strain	Solution Number	X_1 (°C)	X_2 (d)	X_3 (mL)	X_4 (mm)	TAC (%)	Delignification (%)	CL (%)
Untreated		_	_	0	3.2	51.4	0.0	0.0
Tvm4D	1	34.0	35.0	15.0	1.9	63.0	22.5	−14.4
	2	22.0	31.0	5.0	6.4	61.4	13.2	−11.0
TV52J	1	34.0	35.0	15.0	6.4	66.2	14.1	−20.3
	2	22.0	21.0	5.0	3.2	58.1	21.5	−2.6
PC	1	34.0	21.0	15.0	1.6	66.2	42.5	−11.77
	2	22.0	21.0	5.0	6.4	58.2	39.9	2.6

PC = *P. chyrsosporium*; Tv52J = *T. versicolor* 52J; Tvm4D = *T. versicolor* m4D; X_1 = fermentation temperature (°C); X_2 = fermentation time (d); X_3 = inoculum concentration (mL); X_4 = hammer-mill screen size (mm); TAC = total available carbohydrate; CL = cellulose loss.

Contrary to expectation, solid-state cultivation at low severity (solution #2) enhanced the enzymatic digestibility of the substrate more than fungal pretreatment at high severity (solution #1). For Tv 52J, pretreatment at temperature of 22 °C, 21 d fermentation, and 5 mL inoculum concentration released 46.9% more total reducing sugar than that of the pretreatment at 34 °C, 35 d fermentation, and 15 mL inoculum concentration. The results of previous studies have shown that short fermentation time favors enzymatic digestibility for most white rot fungi even though delignification at such a point is often low [58–60]. A study reported that fungi with a high capacity for producing ligninolytic enzyme do not necessarily degrade lignocellulosic biomass proportionately [61]. Moreover, further examination of the physicochemical and ultrastructural changes in fungal-treated switchgrass has shown that, besides lignin removal, an increase in particle pore sizes contributes to enhanced enzymatic hydrolysis [62]. Also, it can be deduced from Figure 4 that a high inoculum concentration does not necessarily suggest efficient solid-state fermentation in

terms of enzymatic digestibility. Hence, solution #2 (optimum conditions) is preferred because it results in a more significant TRS yield and favors a relatively low cost of pretreatment in terms of low fermentation temperature, inoculum concentration, and short fermentation time. Fungal pretreatment time is a major contributor to sugar production cost. A reduction in fermentation time from 60 d to 7 d led to a 33–37% decrease in sugar production cost using grasses, corn stover, and agricultural residues as feedstock [24]. Additionally, a hammer-mill screen size of 6.4 mm (solution #2) gave a higher TRS yield than a hammer-mill screen size of 1.6 mm (solution #1) for *P. chrysosporium*. This implies that solution #2 requires less energy for grinding. A study conducted by Mani et al. [63] showed that it takes about two times the mean specific energy required for grinding switchgrass using a hammer-mill screen size of 3.2 mm than to further reduce it using a hammer-mill screen size of 1.6 mm.

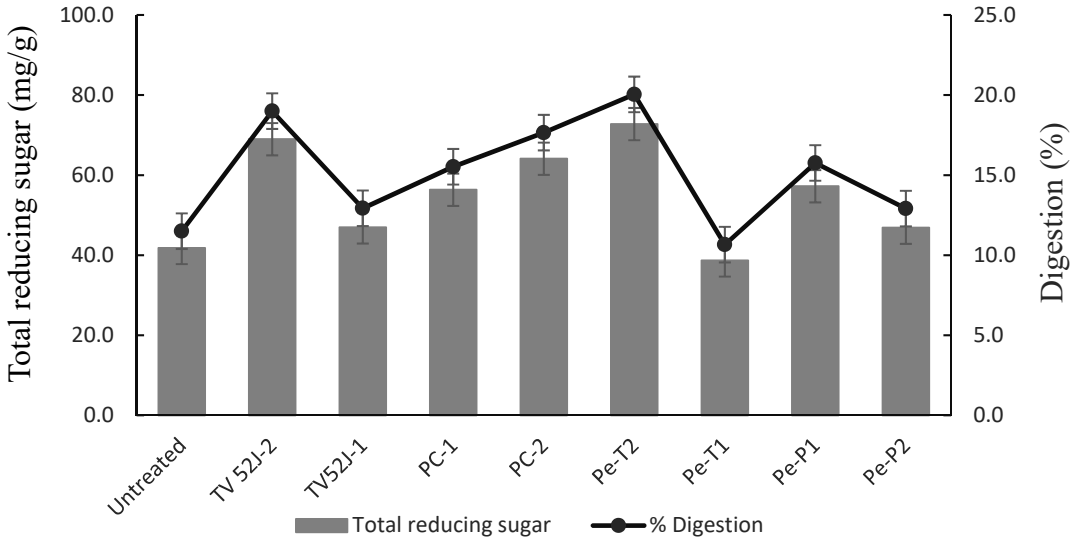

Figure 4. Total reducing sugar yield and percentage of digestion from enzymatic hydrolysis of the fungal-pretreated and untreated switchgrass. Tv 52J-1: *T. versicolor* 52J-treated sample under optimum solution #1; Tv 52J-2: *T. versicolor* 52J-treated sample under optimum solution #2; PC-1: *P. chrysosporium*-treated sample under optimum solution #1; PC-2: *P. chrysosporium*-treated sample under optimum solution #2; Pe-T1: Tv 52J-1 pellet; Pe-T2: Tv 52J-2 pellet; Pe-P1: PC-1 pellet; Pe-P2: PC-2 pellet.

No significant increase in TRS yield was recorded after pelletization of the fungal pretreated switchgrass prior to enzymatic hydrolysis except for Tv 52J-2, which had approximately a 5.5% increase in TRS yield after densification. A notable reduction of about 26.8% in TRS yield was observed in PC-2 after pelletization. Solid-state fermentation of switchgrass using PC- and Tv 52J-enhanced enzymatic digestibility of the switchgrass sample; however, the total reducing sugar yield was generally low compared to some other kinds of biomass pretreatment options. A total sugar yield of 345 mg g^{-1} was obtained from switchgrass subjected to microwave-assisted alkali pretreatment [43], while pretreatment of switchgrass with sodium hydroxide yielded a total reducing sugar of about 453.4 mg g^{-1} raw biomass after enzymatic hydrolysis [64]. Although a higher sugar yield was reported in the above-mentioned studies, they are believed to induce more environmental pollution and generate fermentation inhibitors in higher amounts compared to biomass pretreatment with white rot fungi [65]. Some investigators also reported low sugar yields or a minimal effect of fungal pretreatment on enzymatic hydrolysis output [46,66]. During solid-state fermentation, white rot fungi utilized cellulose hydrolyzed by hydrolytic enzymes as an energy source [29], resulting in cellulose loss. This loss in cellulose is seen as one of the possible reasons for a low hydrolysis yield of fungal-pretreated biomass [60]. Other reports have suggested that toxic metabolites generated by the fungus inhibit the activity of the

hydrolysis enzymes [46,59], hence the low efficiency of enzymatic hydrolysis which is more pronounced at long fermentation time. Kalinoski et al. (2017) attempted to improve the output of enzymatic hydrolysis of fungal-treated miscanthus by adding Glucanex to the enzyme cocktail (Celluclast and Novozyme 188) at the same treatment conditions, which resulted in a significant increase in glucose release for Tv 52J and a reduction in glucose yield for the mutant strain (Tvm4D). On the other hand, biological delignification of rice straw and herbaceous weed *Parthenium* sp. prior to enzymatic saccharification resulted in a reduction in the sugar yield by approximately 455–509 mg/gdw [67]. While a significant positive impact of solid-state cultivation using white rot fungi on enzymatic digestibility was reported in some studies [68,69], a negative effect of fungal pretreatment on enzymatic hydrolysis was noted by investigators like Shi et al. (2009) [33], who reported a glucose yield of 55.6 mg g^{-1} of cotton stalks pretreated with *P. chrysosporium*, which was approximately 17% lower than the yield of untreated cotton stalks after enzymatic hydrolysis in spite of significant lignin degradation. The variations in fungal species, fungal treatment conditions, experimental parameters for hydrolysis, and enzyme diversity adopted in different studies resulted in discrepancies in fungal pretreatment efficiency and, consequently, make the comparison of hydrolysis yield from this present study and other works difficult. Nevertheless, the best treatment conditions for solid-state fermentation of switchgrass using *P. chrysosporium* and *T. versicolor* 52J at low severity with a positive impact on enzymatic digestibility was established in this study. The present study has demonstrated that the use of statistical modelling can determine the optimal conditions for fungal pretreatment that maximizes its advantages while obviating some of the limitations of the approach.

3.7. Characterization of the Fungal Treated Switchgrass and Its Pellets

The physical and mechanical properties of pellet from switchgrass pretreated at optimum fungal pretreatment conditions (solution #2) were evaluated to assess pellet quality. The results of the physical and mechanical properties of the untreated and fungal-treated switchgrass pellets are shown in Table 5. Pellet tensile strength, porosity, and unit density from switchgrass pretreated with PC and Tv 52J were lower than in the pellet from the untreated sample. The poor quality of the fungal-treated switchgrass pellets is further illustrated in Figure 5. The 3D computer tomography images reveal the porosity of the pellets, with the light blue portions of the image representing pore space in the pellet. The Tv 52J-treated pellets had the most pores, which were approximately 28.3% of the pellet. This observation agrees with the report of our previous study [31] that short fermentation time does not improve pellet mechanical strength, which suggests that inherent binders in lignocellulosic biomass are released at longer fermentation times. The longer the fermentation time, the more the lignin and holocellulose are being decomposed by the fungus, which facilitates the bonding of particles in the pellet. This assertion aligns with the work of Kalinoski et al. [46] and Gao et al. [70], which reported an improvement in pellet mechanical strength after 84 d and 35 d of fermentation of the feedstock, respectively. In contrast to enzymatic digestibility, fungal treatment at optimum fungal pretreatment conditions (solution #2) had a negative impact on the switchgrass pellet quality vis-à-vis its handling and transportation. This further indicates that the physicochemical and structural conditions required for optimum enzymatic hydrolysis of biomass differ from those needed for the production of pellets with good quality. Onu et al. [31] demonstrated that the quality of switchgrass pellets was enhanced at optimum fungal pretreatment conditions, which are of higher severity than that reported in the present study. The pretreatment of switchgrass with white rot fungi at low severity conditions considered in the present study did not improve switchgrass pellet quality. However, a study on the optimization of fungal pretreatment of switchgrass using white rot fungi for improved pellet quality, reported elsewhere [31], showed that fungal pretreatment at certain pretreatment conditions improved the quality of switchgrass pellets. Further studies on the technoeconomic assessment of

fungal treated switchgrass pellet production and use should be conducted to assess the cost implication of the application of fungal pretreatment in pellet production.

Table 5. Physical and mechanical properties of fungal-treated and untreated switchgrass pellets.

Sample	Unit Density (kg m^{-3})	Relaxed Density (kg m^{-3})	Tensile Strength (MPa)	Porosity (%)
Untreated	1075.01 ± 81.25	984.36 ± 113.54	1.03 ± 0.28	15.7
Pe-T2	987.19 ± 66.58	947.24 ± 62.03	0.65 ± 0.28	28.3
Pe-P2	898.45 ± 99.94	852.34 ± 52.56	0.87 ± 0.19	16.7

Pe-T2 = *T. versicolor* 52J treated (optimum solution #2) switchgrass pellet; Pe-P2 = *P. chrysosporium* treated (optimum solution #2) switchgrass pellet.

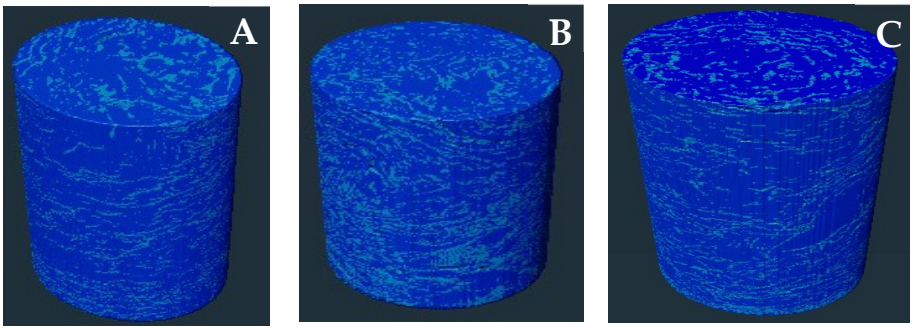

Figure 5. 3D computed tomography images of fungal-treated and untreated images. (**A**) = untreated switchgrass pellet; (**B**) = *T. versicolor* 52J treated pellet; (**C**) = *P. chrysosporium* treated pellet.

The elemental composition of the untreated and fungal-treated switchgrass grinds is presented in Table 6. Minimal changes in the elemental composition of the fungal-treated samples with respect to the untreated were observed. The percentage of ash content in the control sample (untreated) was found to be 17.5% higher than that in the Tv 52J-treated sample and 16.3% lower than the PC-treated sample. The ash content of switchgrass pretreated with fungal strains reported in this present study differs slightly with the result of our previous study on fungal-treated switchgrass pellets [31]. The variation in percentage of ash content is probably related to the different pretreatment conditions used in the two studies. The percentage composition of biomass at varying pretreatment conditions is often not the same.

Table 6. Elemental composition and ash content of fungal-treated and untreated switchgrass.

Sample	Nitrogen (%)	Carbon (%)	Hydrogen (%)	Sulfur (%)	Ash Content (%)	Oxygen (%)
Untreated	0.25 ± 0.01	44.98± 0.49	6.18 ± 0.08	0.05 ± 0.03	3.25 ± 0.43	45.29
Tv 52J-2	0.33 ± 0.02	44.42 ± 0.15	6.10 ± 0.01	0.07 ± 0.00	2.68 ± 0.70	46.40
PC-2	0.22 ± 0.06	44.47 ± 0.13	6.09 ± 0.00	0.16 ± 0.00	3.78 ± 0.10	45.28

Tv 52J-2 = *T. versicolor* 52J treated sample under optimum solution #2; PC-2 = *P. chrysosporium* treated sample under optimum solution #2.

The mineral composition of the ash of the switchgrass samples presented in Table 7 shows that basic elements in the ash ranged between 10.63 and14.40 mg g^{-1}, while heavy metals ranged between 0.17 and 0.25 mg g^{-1}. Calcium, potassium, magnesium, phosphorous, and sulfur were the dominant macro elements present in the ash, with calcium having the highest share of the total weight of all the elements. Consequently, there is a low likelihood of having a slagging problem in combustion systems using switchgrass pellets despite its high ash content in comparison to woody biomass. The mineral composition of ash and its combustion temperature have been identified as the root causes of fouling and slagging rather than the percentage of ash content [71]. The presence of calcium as a major

element in switchgrass ash lowers the risk of slagging in combustion systems because of its high melting point. The fouling and slagging indexes of switchgrass and hardwood were assessed, and the result indicated that switchgrass has a lower potential for fouling and slagging in biomass combustion systems [72]. With the significant concentration of K, P, Ca, and S, and low concentration of heavy metals in fungal-treated switchgrass ash, it can serve as fertilizer for agricultural purposes. This study clearly demonstrated that the solid-state fermentation of biomass using white rot fungi can enhance the enzymatic digestibility of cellulose. It was also established that the solid-state fermentation of biomass at short fermentation times is more likely to enhance enzymatic saccharification than long fermentation times. The reduction in fermentation time is believed to positively impact the cost of fungal pretreatment. Additionally, the findings of this study have shown that different chemical and structural changes in lignocellulosic biomass are required for enhanced enzymatic digestibility and improved pellet quality.

Table 7. Mineral composition (mg g^{-1}) of ash from untreated and fungal-treated switchgrass.

Elements	Untreated	Tv 52J-2	PC-2
		Basic Elements	
K	3.78	2.42	3.92
Ca	4.26	4.30	5.11
Mg	1.47	1.36	1.74
Na	0.07	0.09	0.10
P	1.38	1.36	2.04
S	1.13	1.01	1.38
Al	0.07	0.09	0.11
Subtotal	12.16	10.63	14.40
		Heavy Metals	
Fe	0.119	0.099	0.147
Cu	0.008	0.005	0.007
Zn	0.025	0.022	0.045
Mn	0.025	0.029	0.036
Pb	0.000	0.001	0.001
Cr	0.001	0.001	0.001
Co	0.003	0.001	0.001
Mo	0.004	0.004	0.003
Ni	0.005	0.005	0.005
Subtotal	0.19	0.17	0.25
Total	12.35	10.79	14.65

Tv 52J-2 = *T. versicolor* 52J treated sample under optimum solution #2; PC-2 = *P. chrysosporium* treated sample under optimum solution #2.

4. Conclusions

A study on the optimization of solid-state fermentation using white rot fungi and switchgrass as feedstock was conducted. The ANOVA shows that, for the SSF of switchgrass with PC and Tv 52J, fermentation temperature had a significant ($p < 0.05$) effect on the response variables studied, while fermentation time was more statistically significant for pretreatment with Tv m4D. Fungal pretreatment under low severity (22 °C and 21 d), which is relatively energy-saving and cost-effective, resulted in a higher yield of total reducing sugar than pretreatment at high severity (34 °C and 35 d). Among the white rot fungi, Tv 52J led to a significant increase in the percentage of digestion of cellulose, which further increased by 5.5% after pelletization. This study has shown that longer fermentation times negatively impacted the output of enzymatic hydrolysis despite the high delignification that is associated with long fermentation times. Additionally, the optimum fungal pretreatment conditions favorable to enzymatic digestibility have an opposite effect on the quality of switchgrass pellets. Fungal pretreatment can enhance the enzymatic digestibility of lignocellulosic biomass with minimal environmental burden, but the fermentable sugar yield may not be sufficient for a sustainable cellulosic ethanol

production. However, further investigations on biological delignification kinetics and the interactive effect of fungal metabolites and hydrolytic enzymes on enzymatic digestibility are needed for improved process optimization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fuels3040043/s1, Table S1: Percentage total available carbohydrate, cellulose loss, delignification, and selectivity value of fungal treated switchgrass.

Author Contributions: Conceptualization: O.O.O., L.G.T. and T.D.; methodology: O.O.O., L.G.T. and T.D.; software: O.O.O.; validation: O.O.O., L.G.T. and T.D.; formal analysis: O.O.O.; investigation: O.O.O.; resources: O.O.O., L.G.T. and T.D.; data curation: O.O.O.; writing—original draft preparation: O.O.O.; writing—review and editing: O.O.O., L.G.T., T.D., E.M. and D.C.; visualization: O.O.O.; supervision: L.G.T. and T.D.; project administration: L.G.T. and T.D.; funding acquisition: L.G.T., T.D., E.M. and D.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Biofuel Network (BFN) (ASC-16) and Natural Sciences and Engineering Research Council Canada (NSERC) (RGPIN-2017-05287). We also acknowledge the funding support from the University of Saskatchewan (CGPS Dean's Scholarship).

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to Yousef Papadopoulos, Research Scientist, Science and Technology Branch (Agriculture and Agri-Food Canada) for providing switchgrass (*Panicum virgatum* L.) variety "Cave-in-rock" used in this study. The switchgrass variety is part of Papadopoulos' biomass research program located at AAFC's Nova Scotia Research and Development Centre (Nappan Research Farm, Nova Scotia, Canada) under AAFC's Clean Technology Program.

Conflicts of Interest: The authors declare no conflict of interest.

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