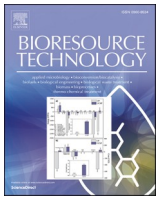
Bioresource Technology 344 (2022) 126256

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



Shiitake cultivation as biological preprocessing of lignocellulosic feedstocks – Substrate changes in crystallinity, syringyl/guaiacyl lignin and degradation-derived by-products

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HIGHLIGHTS

- Three wood species combined with whey additions were designed for shiitake substrate.
- Whey affected more fungal production and lignocellulose degradation than wood species.
- Low nitrogen maximise delignification but minimise glucan degradation.
- Delignification was correlated with reduction of syringyl-to-guaiacyl ratio.
- Slight changes in substrate crystallinity but phenolics increased up to seven-fold.

ARTICLE INFO

Keywords:
Fungal pretreatment
Hardwood substrate
Lignocellulose degradation
Phenolic components
PLS model

ABSTRACT

Formulation of substrates based on three hardwood species combined with modulation of nitrogen content by whey addition (0–2%) was investigated in an experiment designed in D-optimal model for their effects on biological preproceesing of lignocellulosic feedstock by shiitake mushroom (*Lentinula edodes*) cultivation. Nitrogen loading was shown a more significant role than wood species for both mushroom production and lignocellulose degradation. The fastest mycelial colonisation occurred with no nitrogen supplementation, but the highest mushroom yields were achieved when 1% whey was added. Low nitrogen content resulted in increased delignification and minimal glucan consumption. Delignification was correlated with degradation of syringyl lignin unit, as indicated by a significant reduction (41.5%) of the syringyl-to-guaiacyl ratio after cultivation. No significant changes in substrate crystallinity were observed. The formation of furan aldehydes and aliphatic acids was negligible during the pasteurisation and fungal cultivation, while the content of soluble phenolics increased up to seven-fold.

1. Introduction

Forest residues, wood-processing by-products and other lignocellulosic materials are promising renewable resources for bioconversion to advanced biofuels and platform chemicals. That would allow reducing environmental problems caused by the use of fossil resources and supporting bioeconomy development. Enzymatic saccharification of cellulose is crucial in lignocellulose bioconversion, since it produces sugars that can then be converted to bio-based products. However, due to feedstock recalcitrance, enzymatic saccharification of raw lignocellulose results in low rates and yield (Shirkavand et al., 2016; Wang et al., 2018). Pretreatment is required to reduce biomass recalcitrance and to facilitate enzymatic saccharification (Jönsson and Martín, 2016; Zhu and Pan, 2010). By removal lignin or hemicelluloses, or other actions on biomass chemistry and structure, pretreatment reduces feedstock recalcitrance (Zhao et al., 2012).

Many pretreatment approaches have been investigated (Galbe and Wallberg, 2019), and the research on different materials has shown that the effectiveness of a given method is feedstock-dependent (Martín, 2021). Biological pretreatment, consisting in lignin removal by lignin-

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https://doi.org/10.1016/j.biortech.2021.126256
Received 7 September 2021; Received in revised form 26 October 2021; Accepted 27 October 2021
Available online 1 November 2021

degrading microorganisms, has attracted researchers' interest because it has low input of energy and chemical use (Wan and Li, 2012). Drawbacks of conventional biological pretreatment are its slow rate and high rate of cellulose consumption. Recent studies, showing the possibility of combining biological pretreatment with production of edible fungi bring back the attention to this method (Xiong et al., 2019).

The example of shiitake (*Lentinula edodes*), a white-rot fungus, which is the most cultivated edible mushroom in the world (Royse et al., 2017), is rather explicit. After growing shiitake on wood, the spent mushroom substrate (SMS) is delignified to a large extent (Wei et al., 2020) as a result of the fungal activity during cultivation. Due to its low lignin content, SMS is less recalcitrant to a bioconversion to fermentable sugar for ethanol production than the initial substrate. Such a concept of integrated production of bioenergy (ethanol) and food (edible mushroom) suits the bio-based circular economy concept. In order to develop this concept towards industrial implementation, basic understanding on the fundamental mechanisms behind the reduction of the recalcitrance is necessary.

Biomass properties vary considerably between species (Kan et al., 2016), which may result in different degrees of fungal degradation. Lignin, in close association with polysaccharides in lignin-carbohydrate complexes, and its composition and structure play a key role in blocking enzyme access to cellulose (Jönsson and Martín, 2016; Shirkavand et al., 2016). Syringyl (S) and guaiacyl (G), are the main units of lignin macromolecule, and their relative fractions varied with tree species (Wang et al., 2018). A low S:G ratio is believed to cause higher non-productive adsorption of cellulases to lignin, and thus a poorer enzymatic hydrolysis, than a high S:G ratio (Guo et al., 2014). Thus, whether biological pretreatment of woody biomass using shiitake affects lignin S:G ratio, and how it does for different tree species is an important question to be explored. Wang et al. (2018) reported that sulfuric-acid-pretreatment decreased the S:G ratio of lignin, and that was more remarkable for aspen and birch than for spruce. Santos et al. (2012), on the other hand, found that kraft pretreatment removed relatively more G-lignin in several hardwood materials, including *Alnus rubra*, *Eucalyptus urograndis*, *Quercus rubra* and *Acacia mangium*. However, to the best of our knowledge, no experimental research on changes of lignin S:G ratio following wood treatment with shiitake has been reported.

A drawback of several conventional thermochemical pretreatments is the occurrence of side reactions resulting in formation of by-products, such as furan aldehydes, aliphatic acids and phenolic compounds, which are potential inhibitors of cellulolytic enzymes and fermenting organisms (Jönsson and Martín, 2016; Kim et al., 2013). Since biological pretreatment is performed under mild conditions, the formation of most inhibitors that are typical of thermochemical pretreatments might not be expected. Anyway, formation of phenolics and acetic acid might result from the fungal degradation of lignin and hemicelluloses, respectively. Furthermore, as heating is used for pasteurisation, which is essential for substrate preparation, the formation of inhibitors might be possible. Another concern is how different the formation of inhibitors could be for substrates based on diverse tree species.

Wood cellulose has a high degree of crystallinity (Fengel and Wegener, 1989), which is a factor contributing to substrate recalcitrance, and often associated with low reactivity to enzymatic saccharification (Shirkavand et al., 2016; Zhu et al., 2008). Crystallinity changes, attributed to the selective degradation of lignin and hemicelluloses, have been reported after biological pretreatment of lignocellulose. In a comparison of 12 studies on pretreatment with white-rot fungi, the crystallinity incresed in six reports, decreased in three, while in other three almost no change was observed (Shirkavand et al., 2016). Since those reports covered non-edible fungal species grown on and different substrates, it is difficult to conclude if the divergence was caused by differences in fungal species or substrate difference.

Fungal pretreatment is performed by lignin-degrading enzymes secreted by the fungi that colonize lignocellulose. Depending on cultivation conditions, including the nutritional status, fungi can secrete different types and quantities of enzymes and thus results in different outcomes in terms of lignocellulose degradation. Nitrogen, which is often added as nutritional supplement, is important not only for production of edible mushroom bodies (Koutrotsios et al., 2014), but also for the lignocellulose degradation. In previous studies (Chen et al., 2020a; Xiong et al., 2019), birch-based substrates with as low as 0.5–0.6% DM of nitrogen content led to a significantly higher lignin degradation (60–70% of initial mass) and glucan recovery (~70%) than that achieved with a higher nitrogen content (≥0.8%). Nitrogen-limited conditions enhanced lignin-degrading enzymes, and that resulted in more delignification. However, which of S-type or G-type lignin would be more degraded by white-rot fungi, and how this is affected by interaction between substrate species and nitrogen loadings is a question that remains to be investigated. It is also interesting to explore how initial substrate composition and different nitrogen contents would affect the consequent change in substrate crystallinity and formation of degradation by-products by edible white-rot fungi.

In this work, an experiment designed in D-optimal model with combined formula and quantitative factors was performed. Three hardwood substrate species were treated as formula factor and three levels of whey addition as quantitative factor. The parameters (yield and cultivation duration) of shiitake mushroom production and substrate properties (composition of carbohydrates, lignin, substrate crystallinity and lignocellulose-derived by-products) were response variables. The effects of the factors on the response variables were revealed using partial least squares regression (PLS) models, which was expected to be applied for an optimisation of fungal treatment.

2. Materials and methods

2.1. Shiitake cultivation

2.1.1. Substrate materials

Small trees of white birch (*Betula pubescens* Ehrh.), alder (*Alnus incana* (L.) Moench) and aspen (*Populus tremula* L.) with diameter of 4–12 cm at 1.3 m height removed during thinning of a natural forest in Vännäs, Sweden, were used in the experiment. The stems without branches and top materials were debarked, chipped, dried and finally ground. Wheat (*Triticum aestivum* L.) bran was purchased from the Swedish agricultural cooperative (Lantmännen, Sweden). Whey powder (Whey-100, SHNG AB, Sweden), a by-product of the manufacture of cheese, was used as nitrogen additive. Some features of the substrate ingredients are provided in Table 1.

2.1.2. Experimental design

An experiment composed of 17 runs ("treatments") was designed in D-optimal model with combined formula and quantitative factors using the software MODDE 11.0 (Umetrics AB, Sweden) (Table 2). Firstly, a formulation factor 'sawdust species' referring to the mass fraction of birch, alder and aspen sawdust was created. Secondly, additions of 0, 1 and 2% (w/w) whey as quantitative factor were used to adjust the C/N ratio in the substrates. The whey, being a by-product of cheese industry, was proved as effective nitrogen sources for shiitake growth in the previous study (Chen et al., 2020a). The design incorporated five replicated center points using blends containing equal proportions of all

Table 1
Substrate ingredients and chemical composition.

Parameters	pH	Ash %DM	C %DM	H %DM	N %DM	S:G	Particle size mm
Birch	5.0	0.28	49.8	6.1	0.11	3.8	1.4–2.8
Alder	4.9	0.46	50.2	6.2	0.25	2.4	1.4–2.8
Aspen	4.8	0.42	49.5	6.1	0.11	2.4	1.4–2.8
Wheat bran	5.9	5.71	46.5	6.1	2.6	0.5	≤ 3
Whey	6.3	–	50	–	13.2	–	≤ 0.2

DW, dry weight; S:G, syringyl-to-guaiacyl ratio in lignin.

Table 2
Experimental design and fractions of ingredients in initial substrate.

Treatments	Substrate ingredients % DM			Sawdust species × 100% DM			Nitrogen %
	Wheat bran	Sawdust	Whey	Birch	Alder	Aspen	
N1	20	80	0	1	0	0	0.53
N2	20	80	0	0	1	0	0.64
N3	20	80	0	0	0	1	0.50
N4	20	80	0	0	0.5	0.5	0.64
N5	20	80	0	0.5	0	0.5	0.55
N6	20	80	0	0.5	0.5	0	0.58
N7	19.6	78.4	2	1	0	0	0.91
N8	19.6	78.4	2	0	1	0	1.07
N9	19.6	78.4	2	0	0	1	0.68
N10	19.6	78.4	2	0	0.5	0.5	0.84
N11	19.6	78.4	2	0.5	0	0.5	0.80
N12	19.6	78.4	2	0.5	0.5	0	0.88
N13	19.8	79.2	1	0.333	0.333	0.333	0.72
N14	19.8	79.2	1	0.333	0.333	0.333	0.68
N15	19.8	79.2	1	0.333	0.333	0.333	0.70
N16	19.8	79.2	1	0.333	0.333	0.333	0.71
N17	19.8	79.2	1	0.333	0.333	0.333	0.74

three tree species, with 1% (w/w) whey.

The substrates were prepared by mixing all ingredients, and then adding water up to a moisture content of 65% (wet based). The pH of the substrates was adjusted to approximately 6.3–6.5 by adding 1% (w/w) $CaCO_3$ of substrate DM. Four replicates were used for each run.

2.1.3. Mushroom cultivation and sampling

Substrate pasteurisation (85 °C for 4 h), incubation and mushroom harvest (first flush only), as well as fresh mushroom yield determination, was performed as previously described (Chen et al., 2020a). When the entire block was fully covered with white mycelia, judged by a visual observation, the colonisation period was considered complete. Samples for chemical analyses were collected from (1) initial substrates right after pasteurisation, and (2) SMSs (all material remaining in each container) right after the harvest of fruit bodies. Initial substrates and SMSs were dried at 45 °C, then milled to ≤ 0.5 mm, and stored in air-tighten plastic bags at room temperature. Before the chemical analysis, the replicated samples of each treatment were proportionally pooled (20% of every replicate by weight) into one mixed sample.

2.2. Characterisation of substrates

2.2.1. Analysis of nitrogen and pH in the substrate

Total nitrogen and total carbon contents were determined using an elemental analyzer-isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Germany). A SensION PH31 pH meter was used to determine pH values.

2.2.2. Compositional analysis

Determination of extractives (water and ethanol) and structural components in the substrates was performed using NREL methods (Sluiter et al., 2008; Sluiter et al., 2005). Analytical acid hydrolysis (AAH) was applied for determination of lignin and carbohydrates. Klason lignin was determined gravimetrically as the AAH residue, and acid-soluble lignin in the hydrolysates was determined spectrophotometrically at 240 nm (Shimadzu, Kyoto, Japan). Glucose and xylose in the hydrolysates were analyzed with HPLC (High-Performance Liquid Chromatography), using an Aminex HPX-87H column and an RI detector. Elution was performed with isocratic flow of a 5 mM aqueous solution of sulfuric acid. The flow rate was 0.6 mL/min and the column temperature was set to 55 °C.

The relative mass change of each component was determined using mass balances

$$\text{Relative mass degradation}\% = \frac{1-(M_{SMS}\times C_{SMS})}{M_{INI}\times C_{INI}}\times 100$$

where M and C refer, respectively, to mass of substrate and content of component (extractives, glucan, xylan, lignin or soluble phenolics) of SMS and initial (INI) substrates, respectively (Equation. 1).

2.2.3. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analysis

Py-GC/MS was used to determine the relative ratio of dimethoxylated (syringyl, S) and monomethoxylated (guaiacyl, G) lignin units in raw substrate ingredients, initial substrates and SMSs. The analysis was performed at Umeå Plant Science Centre (Umeå, Sweden) according to the method described by Gerber et al. (2012).

2.2.4. Determination of degradation-derived by-products

For determining the possible formation of degradation products, liquid media were prepared by suspending aliquots of the initial substrates and SMSs in 50 mM sodium citrate buffer (pH 5.2) at 10% solids content in the same way as used for enzymatic hydrolysis (Gandla et al., 2018). The suspensions were vortexed and then incubated for 2 h. After that, the liquid phase was separated by centrifugation, acetic acid, formic acid and levulinic acid were determined by HPAEC-PAD (Dionex ICS-5000, Sunnyvale, CA, USA) using a 4 × 50 mm AG15 guard column and a 4 × 250 mm AS15 separation column (Dionex) and a conductivity detector set at 35 °C. A 10 mM aqueous solution of sodium hydroxide at a flow rate of 1.2 mL/min was used as eluent, as previously indicated (Martín et al., 2019). Possible presence of the furan aldehydes furfural and 5-hydroxymethylfurfural (HMF) was evaluated with an HPLC system (Dionex UltiMate 300; ThermoFisher, Waltham, MA, USA) with a diode-array detector and a 3 × 50 mm, 1.8-μm Zorbax RRHT SB-C 18 column. The temperature was set to 40 °C.

For determination of soluble phenolics, boiling water extraction was performed for 3 h. Soluble phenolic compounds in the extracts were determined by Folin Ciocalteu's method (Singleton et al., 1999) using vanillin as calibration standard.

2.2.5. X-ray diffraction (XRD) analysis

To determine the crystallinity index (CrI) of cellulose in the studied samples, powder X-ray diffraction (XRD) analyses were performed using a Malvern Panalytical X'Pert^3 Powder diffractometer equipped with an Empyrean Cu LFF HR X-ray generator and a X'Celerator detector. The patterns were acquired by exposing the samples to Cu Kα1 radiation (1.54056 Å). The instrument was operated at 1.8 kW and the samples were scanned in the 2 θ range between 5 and 45°. The crystallinity index

was calculated by Kumar et al. (2009), based on the height ratio between the intensity of the crystalline peak and the total intensity after the background signal (non-crystalline) using the expression

$$\mathrm{CrI} = \frac{I_{200} - I_{non-cr.}}{I_{200}} \times 100$$

where I_{200} is the maximum intensity of the peak corresponding to the plane in the sample with the Miller indices 200 at a 2 θ between 22 and 24°; $I_{non\text{-}cr}$ is the intensity of the non-crystalline material, which is taken at an angle of aproximatelly 18° 2 θ in the valley between the peaks (Equation 2).

2.3. Statistical analysis

The influence of whey additions and tree species was studied in an experiment with a D-optimal factorial design and its response variables were evaluated by partial least squares (PLS) regression using the software MODDE 11.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden). R^2 and Q^2 values were used to indicate the goodness of fit and predictive ability of the model, respectively; values close to 1 indicated that the model fits the data completely. Q^2 is considered good when it is above 0.5, and the difference between R^2 and Q^2 is lower than 0.2–0.3. A Q^2 value above 0.1 is considered as significant (Eriksson et al., 2008). The number of factors used in the models was determined by optimization of Q^2. The interaction terms showing no significant effect on the target response variable ($p > 0.05$) were excluded. Principal component analysis (PCA) was performed to gain an overview of the data using SIMCA 14.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden). Bivariate correlation followed by Pearson test was conducted to analyse the correlation between data using SPSS statistical analysis software (IBM SPSS version 26.0).

3. Results and discussion

3.1. Fungal colonisation and fresh mushroom production

After some days of inoculation of shiitake mycelium, colonisation signals started to be evident in most of the experimental runs with formulated substrates of either single species ingredient or two- or three-species mixtures. The colonisation pattern was variable for different substrates, and it was sensitive to the tree species used and to the whey addition. It should be noted that one of the experimental runs (N7, birch based substrates with 2% whey, corresponding to 0.91% N) suffered from unexpected termination of the colonisation around two–three weeks after inoculation. Repeated trials (5 × 4) ended up the same results. Based on the experimental results, PLS regression models were developed. The observations of the experimental run N7 were excluded as missing data in the modelling, which was confirmed as valid in terms of the statistical program used. The PLS models revealed the effect of both wood species and whey addition on the colonisation time by shiitake mycelia (Fig. 1a-c) and on the fresh mushroom yield (Fig. 1d-f). The colonisation time exerted significant effect according to Q^2 values, and the whey addition was more influential than the tree species used as substrate. The full colonisation took 18.3–27.8 days for the different experimental runs. A significantly faster ($p < 0.001$) colonisation was observed in the substrates with lower whey additions, which is in

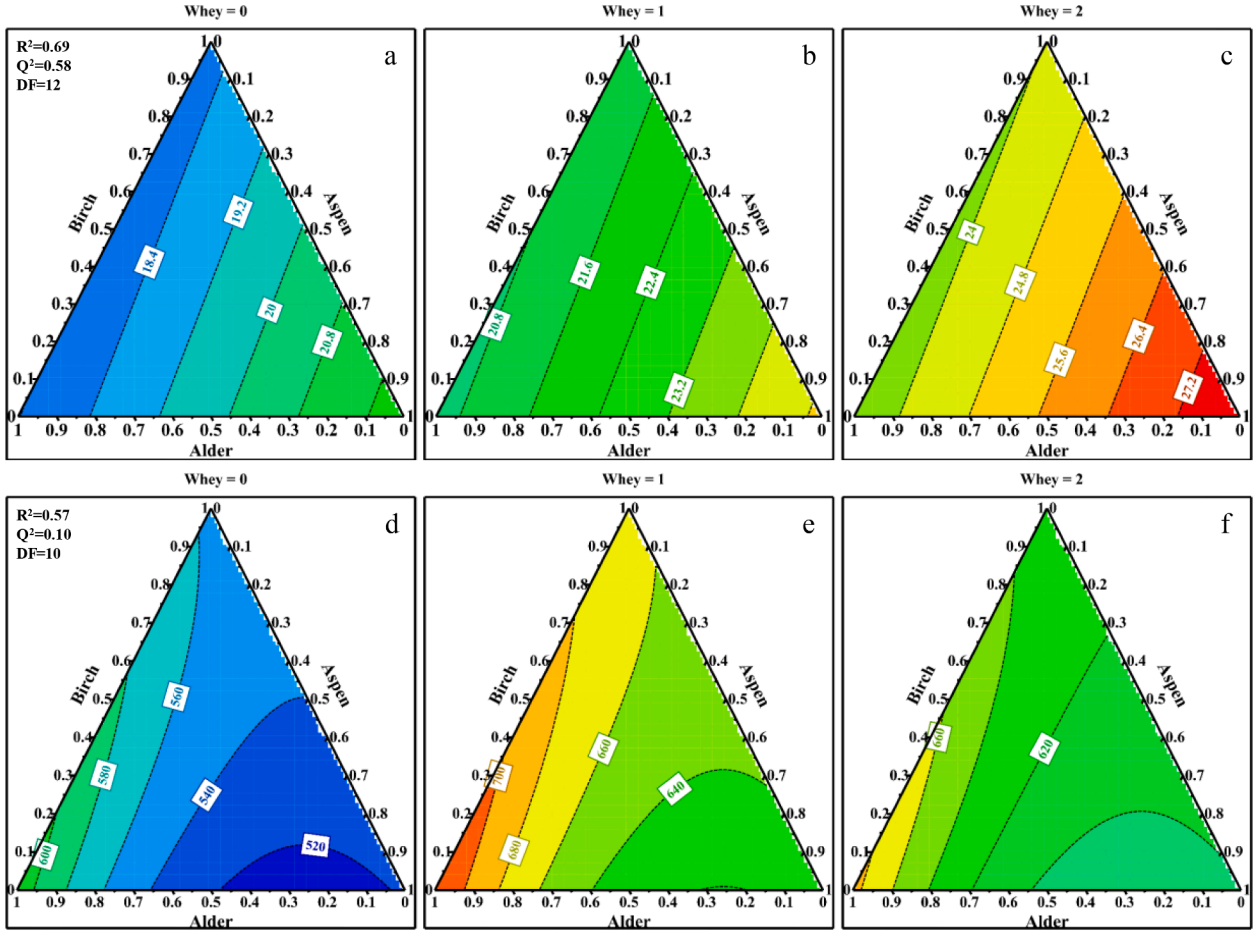

Fig. 1. Response contour plots predicted with PLS regression models for the colonisation time (a-c) and fresh mushroom yield (d-f) in relation to whey addition and tree species. The units are days for the colonisation time and g/kg dry substrate for the mushroom yield.

agreement with a previous study (Chen et al., 2020a). Low fraction of aspen was correlated with a short time of colonisation, in opposite to that of alder, while birch had a milder effect compared with alder and aspen.

Although the entire shiitake cultivation time varied from 74 to 100 days for different experimental runs, no significant effect ($p > 0.05$) of either whey addition or tree species was detected (data not shown).

The yield of the first flush of fresh fruit bodies (with moisture content of 90%) ranged between 520 and 741 g per kg of dry substrate (Fig. 1d-f). The yields were in general comparable to results of previous shiitake studies (650.8–675 g/kg hardwood) (Chen et al., 2020a; Lin et al., 2015; Xiong et al., 2019). Whey additions had a significant ($p < 0.05$) quadratic correlation with mushroom yield; a higher yield was found with 1% whey, followed by 2% and 0% whey addition (Fig. 1d-f). For all whey supplementations, high ratio of alder in the substrate can be associated with high yields of fruit bodies. On the other hand, inclusion of aspen generally resulted in low mushroom yield. However, the Q^2 value was marginally significant for the mushroom yield, and the significance was mostly due to nitrogen loading rather than substrate species.

3.2. Major changes of substrate lignocellulose

3.2.1. Lignocellulose composition

Table 3 shows the contents of major components in the initial substrates and SMSs. The major changes can be generalized as follows: after fungal pretreatment, the contents of lignin (sum of Klason lignin and acid-soluble lignin) and xylan in the SMS were on average 14.4% and 7.4%, respectively. That corresponds to a reduction of 39.6% and 42.8%, respectively, compared with the initial values. On the other hand, average glucan content increased from 31.8% in the initial substrates to 36.9% in the SMSs, which corresponds to around 16% increase.

The compositional changes are evidently associated with the whey additions but not with the tree species (Table 3). The recovered SMS without whey addition had significant ($p < 0.001$) lower lignin content than the ones with 1% and 2 % whey additions. The glucan content in SMS significantly increased with decreasing of whey addition ($p < 0.01$). For the experiments with no whey addition, the nitrogen content in SMS ranged between 0.67 and 0.76%, while for those with whey supplementation of 1 and 2% (w/w), the determined nitrogen content was 0.85% and 0.88–1.13%, respectively. These nitrogen values in the SMSs were higher than those in the initial substrates (Table 2), and they positively correlated ($p < 0.001$) with whey additions.

3.2.2. Lignocellulose mass degradation

Although the previously mentioned changes in the composition of SMSs (Table 3) with respect to the initial substrate point at apparent decreases of the contents of lignin and hemicelluloses, and at increase of glucan content, a better representation is provided by mass balances, which allow calculating the actually recovered and degraded amounts of each biomass constituent. Based on the contents of each component (Table 3), and substrate mass recovery (averaging 64.4% of the starting amount, data not shown) after mushroom cultivation, the relative mass reduction/degradation of major components were calculated (Fig. 2) using the equation 1.

The average mass degradation of lignin, xylan and glucan in the substrates after fungal treatment was 60.4, 61.3 and 26.0%, respectively, in proportion to initial values. Glucan had a much lower rate of degradation than lignin and xylan, and that is consistent with previous studies on birch treatment with shiitake (Chen et al., 2020a; Xiong et al., 2019). The relative mass reductions of lignin and glucan are well described by PLS models with high R^2 and Q^2 values (Fig. 2), which were significantly ($p < 0.05$) affected by the level of whey additions, while the

Table 3
Content of major components in initial substrates and SMSs. Syringil-to-guaiacyl (S:G) ratios of lignin, and crystallinity index are also included. The data refer to mean of duplicate experiments, and the standard errors are included.

Whey	Sawdust species	Glucan		Xylan		Lignin		S:G		CrI		Extractives		S-phenolics		Nitrogen
		Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	Initial	SMS	SMS
%	Bir/Ald/Asp	%	%	%	%	%	%			%	%	%	%	*	*	%
0	1/0/0	28.3	37.0	14.1	8.9	24.1	12.8	2.9	1.6	70	72	10.9	31.2	0.34	2.4	0.74
	0/1/0	30.6	36.7	14.4	8.1	24.7	14.8	2.3	1.2	72.4	71	12.1	29.8	1.56	2.4	0.76
	0/0/1	37.8	39.3	12.1	5.5	22.0	12.5	2.4	1.3	72.3	72.3	9.6	28.8	0.47	2.0	0.74
	0/0.5/0.5	32.7	41.0	12.0	6.6	23.2	13.0	2.1	1.2	70.4	76.8	9.8	29.3	0.80	2.2	0.70
	0.5/0/0.5	30.2	39.7	13.8	7.7	23.1	12.3	2.6	1.4	66.6	73.6	10.6	30.8	0.33	2.2	0.67
	0.5/0.5/0	29.2	38.3	14.7	7.3	24.9	12.8	2.9	1.3	68.9	72	11.0	30.9	0.73	2.1	0.73
1	0.3/0.3/0.3	30.2 ± 0.6	33.5 ± 0.7	12.2 ± 0.2	8.0 ± 0.5	23.7 ± 0.1	15.0 ± 0.4	2.5 ± 0.1	1.5 ± 0.1	69.9 ± 1.3	70.9 ± 1.1	12.0 ± 0.3	29.0 ± 0.6	0.69 ± 0	2 ± 0.1	0.85 ± 0
2	1/0/0	na	na	na	na	na	na	2.8	na	68.3	na	na	na	0.34	na	na
	0/1/0	30.5	29.1	12.3	7.6	24.7	17.6	2.5	1.3	70.9	70.6	13.4	29.4	1.32	2.2	1.13
	0/0/1	36.6	38.9	11.2	7.7	22.5	16.3	2.0	1.6	67.5	73.5	13.0	25.8	0.40	1.9	0.88
	0/0.5/0.5	33.5	31.9	12.4	6.8	24.6	16.2	2.2	1.4	70.8	68	11.6	29.1	0.78	2.0	1.08
	0.5/0/0.5	30.9	38.0	14.2	7.3	23.5	14.6	2.5	1.7	70.9	70.9	11.7	29.6	0.30	1.9	0.89
	0.5/0.5/0	29.6	36.4	11.9	8.5	24.4	15.1	2.6	1.5	69.5	78.5	12.7	30.0	0.65	2.1	1.11
Effects of																
Whey		ns	b	ns	ns	ns	a	ns	b	ns	ns	b	ns	ns	ns	a
Birch		ns	ns	ns	ns	ns	ns	c	ns	ns	ns	ns	ns	ns	ns	ns
Alder		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	a	ns	ns
Aspen		b	ns	ns	ns	ns	ns	c	ns	ns	ns	ns	ns	ns	ns	ns
Whey × Whey		–	c	–	–	–	–	–	–	–	–	–	–	–	–	–
Aspen × Aspen		b	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Bir/Ald/Asp, Birch/Alder/Aspen; Lignin amount corresponds to the sum of the acid-insoluble (Klason) and acid-soluble fractions; CrI, Crystallinity index; S-phenolics, Soluble phenolics; * vanillin equivalent units; na, data not available; a, significant at $p < 0.001$, b, significant at $p < 0.01$, c, significant at $p < 0.05$, ns, non-significant ($p > 0.05$), -, excluded interaction terms ($p > 0.05$; details in section 2.3).

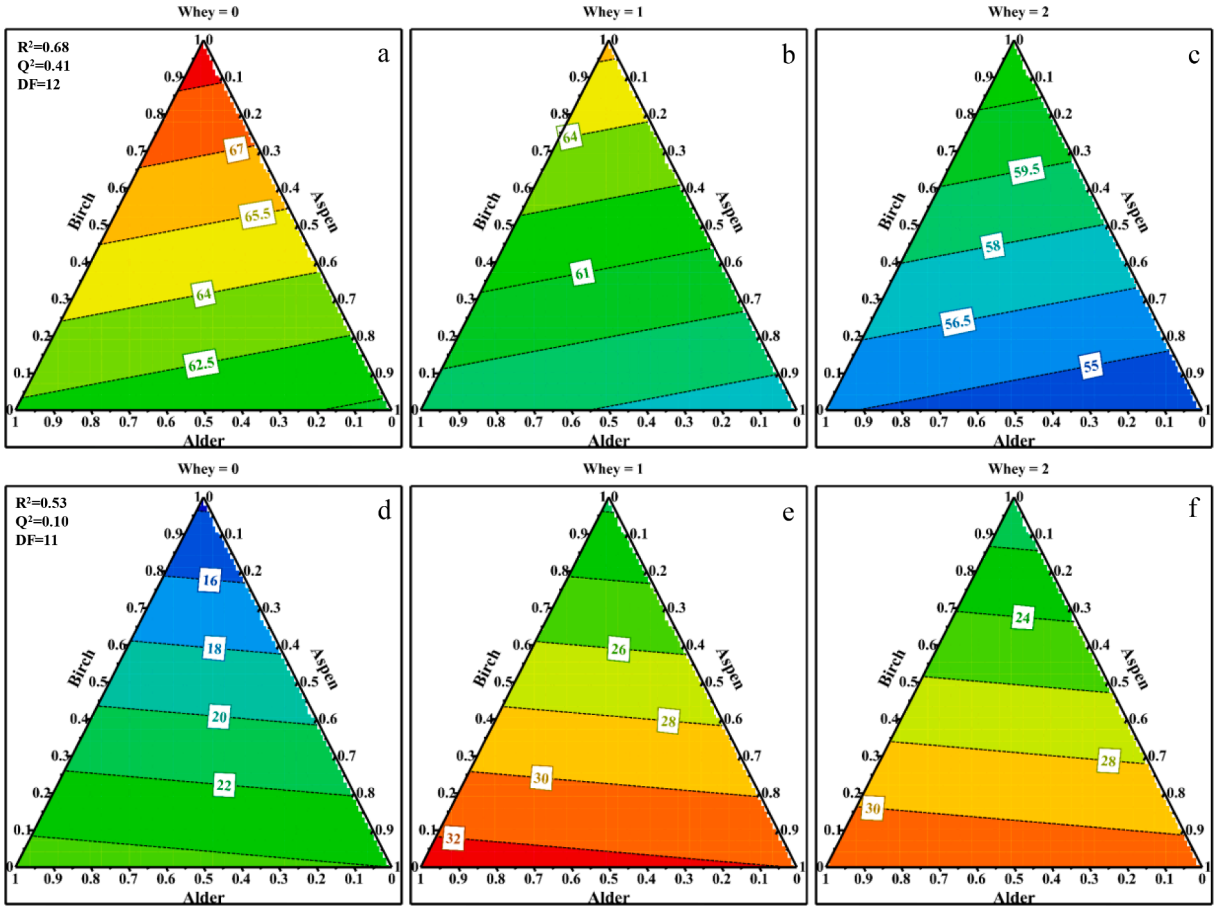

Fig. 2. Response contour plots predicted with PLS regression models for the relative mass reduction (%) of lignin (a-c) and glucan (d-f) in SMSs with respect to the initial substrates.

effect of the wood species used in the substrate was less important.

Lignin degradation was linearly and negatively correlated with whey addition ($p < 0.01$), and showed maximal value of 67.6% with no whey addition (Fig. 2a-c), where the only nitrogen source was that intrinsic of the wood substrates, which corresponded to<0.58% (w/w) (Table 2). Differently, glucan mass degradation was positively correlated with the whey addition ($p < 0.05$) (Fig. 2d-f). Low losses of glucan (16–24%) were observed for the experimental runs without whey addition, while larger losses (24–32%) were found for those with 1% and 2% whey. Although the exact reason for this could not be determined by this study, the nitrogen-rich condition might have increased the growth of competing microbes that consume carbohydrates. Whatever, the fact that maximal degradation of lignin but minimal glucan loss was associated with no addition of whey agrees with what was observed at nitrogen loading of < 0.6% in a previous study (Chen et al., 2020a). Substrate species seemed to be important also, as indicated by contour in Fig. 2: lignin was degraded slightly more in birch than in aspen, in contrast to glucan, which was degraded more in alder than in birch substrate. Compared with those of lignin and glucan, mass reduction of xylan was not significantly ($p > 0.05$; data not shown) affected by either whey addition or tree species.

The high degree of degradation of lignin and xylan, together with the good preservation of glucan, are positive features of the fungal cultivation investigated in this work as a potential pretreatment for enzymatic saccharification of cellulose. The results of the mass balances revealed that glucan recovery as high as 80% can be achieved, which is comparable with the typical range (70–90%) achieved in different lignocellulose thermochemical pretreatment approaches (Ilanidis et al., 2021a; Ilanidis et al., 2021b; Martín et al., 2019; Tang et al., 2021).

3.2.3. *Relative change of syringyl-to-guaiacyl ratio in lignin*

The substrates used for fungal cultivation in this work were based on three hardwood species, namely birch, alder and aspen. Hardwood lignin is composed of a mixture of syringyl and guaiacyl units, but their relative ratios can vary for different species. The raw birch, alder and aspen used for formulating the substrates had syringyl-to-guaiacyl (S:G) ratios of 3.8, 2.4 and 2.4 respectively (Table 1). The higher S:G ratio of birch compared to the other species is in agreement with a previous investigation reporting S:G ratios of 3.3 and 1.7, respectively for birch and aspen (Wang et al., 2018). Another major constituent of the substrates was wheat bran, a byproduct of wheat milling. Wheat bran is composed mostly of carbohydrates and a small lignin fraction. The lignin of the wheat bran used in this work contained 55.6% G-units.

As consequence of the addition of wheat bran, the S:G ratio of the initial substrates was 2.0–2.9, which is lower than the values for the raw wood (Table 3). After the shiitake cultivation, the S:G ratio decreased to 1.2–1.7 in the SMS. The reduction of S:G ratio was 41.5% on average (Fig. 3), which was much higher than the results reported after acid pretreatment of aspen (20.8%) and birch (26.2%) (Wang et al., 2018). The findings in this study were, however, in contrast with reports on alkaline pretreatment showing a general increase in S:G ratio of four hardwood species, which was attributed to a larger proportion of G-lignin been degraded (Santos et al., 2012).

As shown in Fig. 3, whey addition had a significant ($p < 0.001$) effect on the relative change (%) of S:G ratio; high addition resulted in less change of S:G ratio. The effect of the different wood species was, however, only marginally significant (Table 3). Large changes were associated with higher fraction of alder, and lower changes were associated with more aspen in the substrate, while the influence of birch was minor

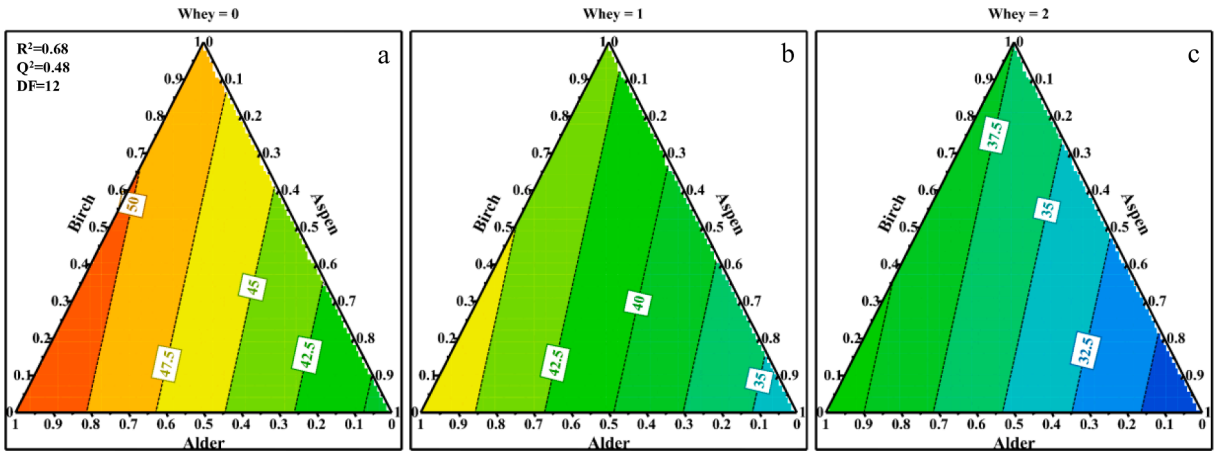

Fig. 3. Response contour plots predicted with PLS regression models for the relative change (%) of the S:G ratio of lignin in SMSs with respect to the initial substrates.

(Fig. 3).

The decrease of the S:G ratio during fungal cultivation might be attributed to a higher enzymatic reactivity of S units in the substrates of this study. It has been reported that both manganese peroxidase (MnP) and laccase, the major lignin-degrading enzymes secreted by shiitake (Janusz et al., 2013), have different preferences regarding degradation of lignin units. For example, MnP generates Mn^{3+} and is capable of oxidizing nonphenolic compounds and minor phenolic moieties of lignin, while laccase is a copper-containing oxidase that oxidizes numerous phenolic compounds (Janusz et al., 2013; Wan and Li, 2012). Wood lignins rich in S units often have low content of free phenolic groups due to their involvement in formation of methoxy groups (Camarero et al., 1999). Plausibly, the high reactivity of S lignin in this study can be attributed to more MnP activity, compared to laccase activity, involved in lignin degradation. For unknown reasons, high nitrogen loading might have caused the repression in MnP activities, and that resulted in even higher ratio of S:G in the SMS and low mass degradation of total lignin. A description of the mechanism is beyond the scope of this study, but it deserves being investigated in future studies including assessment of enzymatic activities.

3.2.4. *Formation of lignocellulose degradation by-products*

Furfural, HMF, levulinic acid and formic acid were not found in any of the samples, whereas acetic acid was not detected in the initial substrates, but it was contained in low amounts in the SMSs (Below 0.1% (w/w), data not shown). Furan aldehydes are typically formed by sugar degradation during hydrothermal treatments in acidic media, and if the temperature is high or the reaction time is long, they can get degraded further to formic acid and levulinic acid (Fengel and Wegener, 1989). The absence of furan aldehydes, levulinic acid and formic acid, indicates that, as expected, hot-air pasteurisation at 85 °C did not cause any major carbohydrate degradation. The absence of acetic acid, which is typically formed from splitting of acetyl groups during xylan hydrolysis (Jönsson and Martín, 2016), in the initial substrates indicates that no hydrolysis of hemicelluloses occurred during pasteurisation. Furan aldehydes and aliphatic acids are known inhibitors of enzymatic saccharification and microbial processes (Jönsson and Martín, 2016). The results presented in this study show that hot-air pasteurisation at 85 °C, and the following fungal cultivation are unlikely to produce these inhibitors if shiitake cultivation would be used as a fungal pretreatment of lignocellulose for bioconversion by enzymatic saccharification and microbial fermentation.

Soluble phenolic compounds were determined in water extracts of the initial substrates and SMSs. As shown in Table 3, the content of soluble phenolics in initial substrates ranged between 0.3 and 1.6% (w/w). Soluble phenolics in the initial substrates are mostly wood extractives (Chen et al., 2020c), such as lignans, isoflavones and other polyphenols. The amount and composition of the phenolics compounds of the wood extractives varies widely from species to species (Valette et al., 2017). In alder-based substrates, a significantly ($p < 0.001$) higher content of soluble phenolics, compared to the other two wood species, was found. A considerable higher content of phenolics was detected in the water extracts of spent substrates after the cultivation. The content of soluble phenolics in the SMS ranged from 1.8 to 2.4% (w/w), which corresponds to 1.4–7.2-fold increases. No significant differences regarding the content of soluble phenolics were found between SMSs resulting from experimental runs with different nitrogen loadings or different tree species. The increased content of soluble phenolics in the SMSs compared with those of initial substrates might be a consequence of formation of relatively small phenolic compounds as result of lignin degradation during fungal cultivation. However, in this work, in the experimental runs with low nitrogen loading, which led to large mass reduction of lignin, the accumulation of soluble phenolics did not seem to be affected significantly (Table 4). The lack of significant correlation between lignin degradation and soluble phenolics might be attributed to the formation of lignin-degradation products that are not water-soluble. Some phenolic compounds are regarded as more toxic compounds towards fermenting microorganisms compared to furan aldehydes and aliphatic acids (Chen et al., 2020c; Jönsson and Martín, 2016). However, since enzymatic hydrolysis is generally performed at 45–50 °C (Gandla et al., 2018), the expected concentrations of phenolics in the hydrolysates are lower than the amounts released by water extraction at boiling temperature. Such a low release of phenolics should probably result in low inhibitory effects in the fermentation.

3.2.5. *Substrate crystallinity*

As presented in Table 3, all initial substrates regardless of tree species had comparable crystallinity indices, and they ranged from 66.6 to 72.4%. The crystallinity in the substrate is mainly due to cellulose, which is a highly crystalline polymer with a compact supramolecular structure stabilized by hydrogen bonds (Fengel and Wegener, 1989). The fungal cultivation did not cause statistically significant changes in the crystallinity. This is in contrast to the proposal that the crystallinity was increased by fungal pretreatment due to a possible retention of crystalline form and more degradation of amorphous forms of cellulose (Shirkavand et al., 2016). However, this agrees with results by Vane et al. (2006), who found that loss of crystalline and non-crystalline regions of cellulose occurred in parallel during growth of shiitake on oak bark during a 101-month cultivation. No significant correlations ($p > 0.05$) were found between lignocellulose degradation and the relative change of crystallinity during fungal pretreatment (Table 4).

Table 4
Correlation analysis of the mass change of substrate major components and relative changes of crystallinity index and S:G ratio.

	Mass change of						Relative change of	
	Block mass	Extractives	Lignin	Glucan	Xylan	S-phenolics	CrI	S:G
Block mass	1	−0.18	0.73b	−0.07	0.44	−0.15	0.00	0.50c
Extractives		1	−0.72b	0.31	−0.66b	0.27	0.10	−0.45
Lignin			1	−0.47	0.64b	−0.29	−0.16	0.68b
Glucan				1	−0.05	0.40	0.44	−0.16
Xylan					1	−0.15	0.31	0.48
S-phenolics						1	0.13	0.27
CrI							1	0.13
S:G								1

b, significant at $p < 0.01$, c, significant at $p < 0.05$.

3.3. Principle components analysis (PCA) and overview of fungal pretreatment using shiitake

An overview of chemical and physical characteristics from all initial substrates and SMSs was performed by a PCA based on data matrix of 10 substrate variables and 32 observations (experimental runs, observations of N7 and NS7 were excluded as missing data). As generalized by the biplot of PC1 × PC2 in Fig. 4, which explains 87.5% of the total variations, the cultivation resulted in distinct divergence of properties from initial substrate cluster (N1-N17) to SMSs (NS1-NS17). The initial substrates revealed an obviously high content of lignin and hemicellulose (see also Table 3), while highly delignified SMS was positively correlated to glucan-rich SMS. The cluster of NS1-NS17 seemed to be more dispersed along PC2 (vertical) axis than that of N1-N17, which was clearly driven by nitrogen and glucan in opposite directions. However, there was no clear pattern that either clustering or dispersion was associated with the tree species. All these are consistent to the direct results from previous sections that nitrogen is more important than the options of studied wood species when the effect of fungal pretreatment is concerned.

In this study, cultivation of shiitake mushroom on hardwood was demonstrated to be a successful model for food production with potential of using the spent substrate for bioconversion to cellulosic ethanol. The achieved yield of fresh fruit bodies (Fig. 1d-f) ensures a good market value. During the cultivation, >60% (w/w) of initial lignin and xylan was degraded (Fig. 2), while as much as 80% of the initial glucan mass remained in the SMS. The high glucan recovery after cultivation, together with the low content of lignin and hemicelluloses of the SMS are indications of the potential of a shiitake-based biological pretreatment for facilitating enzymatic saccharification in a bioconversion process to produce ethanol or other sugar-platform products. Compared with other white-rot fungal species, such as summer oyster mushroom (*Pleurotus pulmonarius*) (Chen et al., 2020b) and wood ear mushroom (*Auricularia auricular-judae*) (Chen et al., 2021), shiitake has

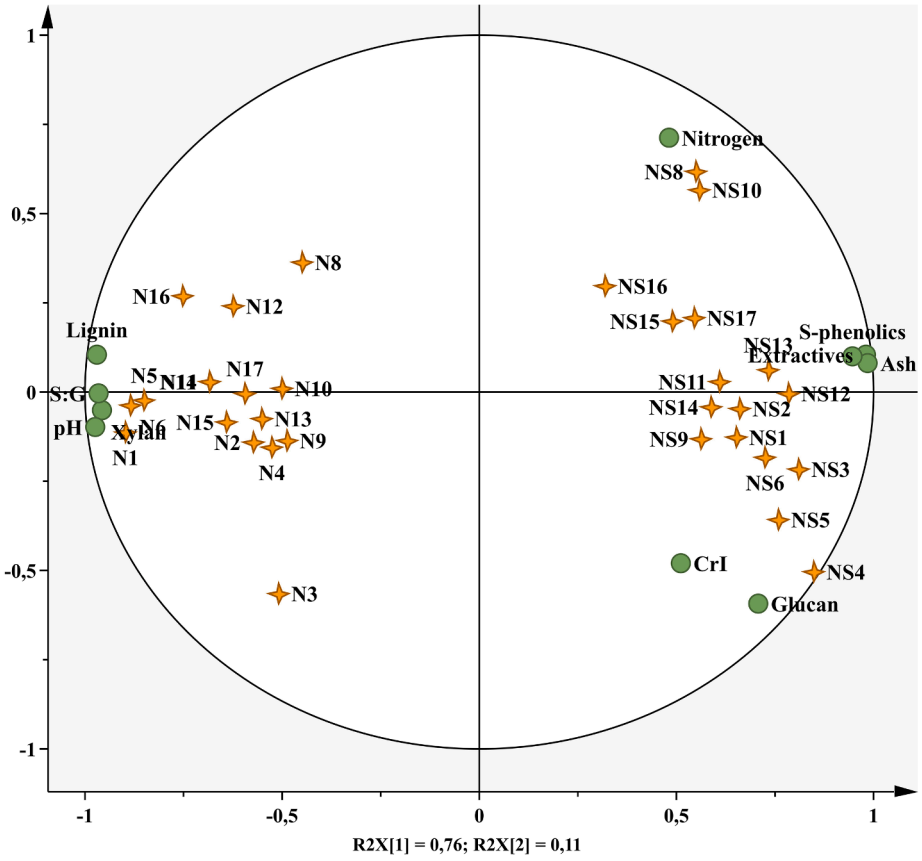

Fig. 4. PCA biplot showing major chemical components of substrates before and after shiitake mushroom cultivation. PCA was based on 32 × 10 matrix of wet chemical data.

a higher ability to selectively degrade lignin and hemicelluloses. It is worth mentioning that in a previous study (Chen et al., 2020a), it was found that glucan losses could be minimized by lowering nitrogen loading in birch-based substrates. In this study, where alder and aspen were included, that finding is proven to be true also for other hardwood species. Furthermore, the negligible formation of inhibitors found in this study reveals an advantage of shiitake cultivation as potential biological pretreatment for lignocellulose bioconversion.

Anyway, a deeper understanding on the mechanisms of lignocellulose pretratment using shiitake is still required. Among other issues, the different susceptibility of S- and G-lignin to degradation and the kinetics of lignin-degrading enzymes remain to be explored. The availabilities of different forms of nitrogen (NH_4^+, NO_3^-, NO_2^-) in the substrate during the fungal cultivation and their effects on the pretreatment should also be investigated. A comprehensive characterisation of the fraction of extractives, which represents as much as 30% of SMS dry mass, including the screening of its composition and the elucidation of the utilisation potential of its components, is required. Although fungal pretreatment resulted in an efficient removal of lignin and hemicelluloses from wood, which is clearly positive for enzymatic hydrolysis, other changes, such as an increase of the share of G-lignin and formation of soluble phenolics were also detected. Elucidating whether and to what extent those phenomena affect the enzymatic hydrolysis are relevant questions to be answered.

4. Conclusion

Shitake cultivation on birch-, alder- and aspen-based substrates resulted in selective degradation of lignin and hemicelluloses, together with good preservation of cellulose. Nitrogen supplementation played a significant regulatory role, and the low levels of nitrogen resulted in fast mycelial colonisation, increased delignification and relatively low glucan consumption. No statistically significant changes in substrate crystallinity were caused by shiitake cultivation. Some formation of phenolic compounds was detected independently on the substrate composition. Favourable features of shiitake cultivation as potential pretreatment for bioconversion of lignocellulose were identified.

CRediT authorship contribution statement

Feng Chen: Investigation, Writing – original draft, Writing – review & editing. **Carlos Martín:** Writing – original draft, Writing – review & editing. **Torbjörn A. Lestander:** Investigation. **Alejandro Grimm:** Investigation. **Shaojun Xiong:** Conceptualization, Project administration, Funding acquisition, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Swedish State Department of Innovation, Swedish State Energy Agency, and Swedish Research Council through the BioInnovation program (VINNOVA 2016-05104, 2017-02705) and Re:Source (P42181) and Bio4Energy (http://www.bio4-energy.se/). The authors would like to thank Carina Jonsson, Gunnar Kalén, Markus Segerström, and Ulla-Britt Östman, SLU (SBT), for laboratory assistance. Dr. Junko Takahashi Schmidt, Umeå Plant Science Centre Biopolymer Analytical Platform, helped with Py-GC/MS analysis. Dr. Roushdey Salh, Department of Physics, Umeå University, helped with XRD analysis.

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