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ORIGINAL ARTICLE

Enabling efficient bioconversion of birch biomass by *Lentinula edodes*: regulatory roles of nitrogen and bark additions on mushroom production and cellulose saccharification

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Abstract
Pretreatment with edible white-rot fungi has advantages in low inputs of energy and chemicals for reducing the recalcitrance of woody biomass for bioethanol production while harvesting protein-rich food. The effectiveness of fungal pretreatment may vary with substrate composition. In this study, birch with or without bark and nitrogen additives were experimentally studied for their effects on shiitake production, substrate lignocellulosic degradation and enzymatic convertibility with cellulolytic enzymes. Whey was added as protein nitrogen and led to successful outcomes, while non-protein nitrogen urea and ammonium-nitrate resulted in mortality of fungal mycelia. The mushroom yields of one harvest were generally comparable between the treatments, averaging 651 g fresh weight per kilogram dry substrate, and high enough as to be profitable. Nitrogen loading (0.5–0.8%, dry mass) negatively affected lignin degradation and enzymatic convertibility and prolonged cultivation/pretreatment time. The added bark (0–20%) showed quadratic correlation with degradation of lignin, xylan and glucan as well as enzymatic digestibility of glucan. Nitrogen loading of < 0.6% led to maximal mass degradation of xylan and lignin at bark ratios of 4–9% and 14–19%, respectively, peak saccharification of glucan at 6–12% and the shortest pretreatment time at 8–13% bark. The designed substrates resulted in 19–35% of glucan mass loss after fungal pretreatment, less than half of the previously reported values. Nitrogen and bark additions can regulate lignocellulose degradation and saccharification of birch-based substrates. The designed substrate composition could considerably reduce cellulose consumption during fungal pretreatment, thus improving bioconversion efficiency.

Keywords White-rot fungi · Biological pretreatment · Birch · Delignification · Enzymatic hydrolysis · Multiple-linear-regression (MLR) model

1 Introduction

Global climatic changes and a growing population call for the increased production of renewable energy. It is estimated that by 2050, around 10–40% of the world's primary energy consumption could be covered by woody biomass [1, 2]. Wood lignocellulose, such as in forest residues, is a potential source of advanced biofuels such as second-generation ethanol. However, the high lignin content in wood (about 20–35% of dry mass) limits enzymatic hydrolysis of cellulose for the production of ethanol [3]. The development of pretreatment technologies, including physical, chemical and biological methods, aimed at reducing biomass recalcitrance to improve enzymatic saccharification of cellulose, for an eventual industrial production of bioethanol, has been a focus of intense academic research in the last two decades [4].

Biological pretreatment using lignin-degrading microorganisms, mainly white-rot fungi, has received research attention due to its low energy input, reduced formation of inhibitors and environmental friendliness [5, 6]. During white-rot fungus growth on woody substrates, lignin is degraded by the oxidoreductases, such as laccases and peroxidases, and used for mycelium formation. This results in a significant decrease of the lignin content and in changes in some physical and biochemical characteristics of the substrate, which improves the efficiency of enzymatic hydrolysis of cellulose [7].

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In spite of the interest within academic research, unless it is combined with other methods [8], biological pretreatment is not yet viable for industrial implementation, due to its slow rate [9] and the partial consumption of carbohydrates by pretreatment microorganisms [10]. A novel concept for biological pretreatment that deserves attention is based on the cultivation of edible white-rot fungi on woody substrates, and shiitake (*Lentinula edodes* (Berk.) Pegler) has been revealed as a promising model species [11]. Although this novel biological pretreatment strategy is still time-consuming and associated with carbohydrate losses, the drawback can be compensated by the co-production of high value-added edible mushrooms. Shiitake is an edible white-rot mushroom that is becoming increasingly popular on the global market because of its flavour, high nutritional content and health-promoting and medicinal properties [12]. Our previous research has revealed that the spent mushroom substrate (SMS) resulting from harvesting shiitake fruiting bodies on woody biomass is a cellulose-enriched material with a low lignin content and enhanced enzymatic digestibility [11]. Pretreatment of woody biomass by shiitake cultivation is also effective in improving anaerobic digestion for biomethane production [13]. However, it is still unclear whether and how cellulose consumption can be reduced while obtaining a high degradation of lignin and a good mushroom yield.

Global shiitake production amounts to 7.5 million tons per year, which corresponds to 22% of the global mushroom market [14]. It is estimated that around 4–5 kg SMS are generated per kilogram of mushroom harvested [15]. This suggests a great potential to integrate shiitake and biofuel production, provided that the cellulose that remained in the SMS is efficiently saccharified and converted to ethanol. Different mushroom producers may use different biomass mixtures for the substrates [16], which may result in differences in lignocellulose degradation and enzymatic digestibility of the spent substrates. An understanding of how the degradation of lignocellulose components during cultivation and the susceptibility of the spent substrate to enzymatic hydrolysis are affected by initial substrate composition is a prerequisite for the further development of a process combining mushroom production and biological pretreatment for lignocellulose bioconversion to ethanol.

Bark constitutes around 10–25% of the dry mass (DM) of a tree stem depending on the species, growing conditions and anatomical part [17]; for birch, it can be 13–20% in correlation with stems/shoot diameters. It is often a major by-product of sawmills where stemwood is processed. Bark typically has lower carbohydrate contents but higher contents of lignin, extractives and ash than stemwood [18]. Although the antioxidant and antimicrobial properties detected in bark extractives may inhibit mycelium growth [19, 20], bark still contains nutrients (minerals) and carbohydrates [21, 22]. However, due to its higher lignin content (33% DM) compared with stemwood (23% DM), based on our pilot analysis, it might be included as an additive to substrates only within a certain percentage range. However, the inclusion of bark in the substrate formulation for shiitake production based on the nutrient-supplemented woodchip method and the effect of initial lignocellulose fractions on changes in substrate composition and the enzymatic convertibility of SMS cellulose have rarely been addressed in the literature.

Nitrogen supplementation is considered a key factor in substrate formulation for industrial mushroom cultivation. Supplying nitrogen of good quantity and bioavailability has been an important topic for both research and industrial practice for mushroom production [23], and the effects may vary with the ratio and source (protein and non-protein nitrogen). The activity of the enzymes involved in lignin degradation may be affected differently [24, 25], probably depending on the bioavailable nitrogen and carbon concentrations. Since the mechanism behind that phenomenon is not yet well understood, additional research is needed to clarify whether the differences in initial substrate composition may have an effect.

This study aimed at a further development of our novel fungal pretreatment process to delignify lignocellulose by cultivation of edible mushrooms on forest residues [11]. The focus of this study was on how substrate formulation could affect the efficiency of substrate delignification and mushroom production, and our hypothesis was that the nitrogen and bark additions in the substrate were important factors for process optimisation. A factorial experiment was designed to investigate the effects of bark and nitrogen addition on (i) mushroom production on a birch-based substrate, (ii) degradation of lignocellulosic components, and (iii) enzymatic digestibility of cellulose contained in the SMS. The potential use of non-protein versus protein nitrogen resources was also evaluated.

2 Materials and methods

2.1 Substrate materials

White birch (*Betula pubescens*) was used as a model species for major substrate material based on our previous study [11]. Birch is frequently found among early forest thinning residues and remains underutilised in the northern hemisphere [26]. Birch trees with a breast diameter of 4–12 cm were freshly harvested from a natural forest area in Vännäs, Sweden, in October 2017. The main stems were split into stemwood and bark by manual debarking. Stemwood and bark were then chipped with an Edsbyhuggen chipper (*Edsbyhuggen* AB, Sweden) to a particle size of 15–20 mm, dried at 45 °C and ground to < 4 and < 5 mm, respectively (Table 1). Wheat (*Triticum aestivum*) bran and barley (*Hordeum vulgare*) grain were supplied by a Swedish food and fodder company

Table 1 Substrate ingredients and chemical composition

Parameter	pH	Ash %DM	Carbon %DM	Hydrogen %DM	Nitrogen %DM	Bulk density kg m^{-3}	Glucan %DM	Xylan %DM	Lignin %DM	Extractives %DM
Birch stemwood (< 4 mm)	5.0	0.3	49.8	6.1	0.1	243.5	37.2	19.9	22.8	4.46
Birch bark (< 5 mm)	4.8	2.2	56.0	6.7	0.5	152.5	16.2	12.3	33.0	22.1
Barley grain (< 8 mm)	5.9	2.1	45.7	6.0	1.6	668.6	55.3	4.4	6.7	20.4
Wheat bran (< 3 mm)	5.9	5.7	46.5	6.1	2.6	238.5	18.0	14.9	12.5	32.6
Whey (< 0.2 mm)	4.6	–	–	–	13.9	–				

(Lantmännen). Whey powder (Whey-100, HSNG AB, Sweden), urea and ammonium nitrate (Sigma-Aldrich) were used as nitrogen additives.

2.2 Experimental design and treatments

A central composite face (CCF) design with two independent factors (bark and nitrogen addition to substrate) was used, each of them at three levels. Three replicated centre points were included, resulting in a total of 11 treatments (Table 2). Each treatment was replicated four times.

To examine the effects of nitrogen on mushroom growth and lignocellulose degradation in different substrates, the treatments were arranged so that the nitrogen content was expected to range from 0.51 to 0.87%, but the total carbon content was around 50% of the DM. Three types of nitrogen additive were studied in three separate experiments: whey (0, 1, 2%), urea (0, 0.5, 1%) and ammonium-nitrate (1, 2, 3%). The doses of nitrogen additive were chosen based on the nitrogen contents of all substrate ingredients; thus, each of the three doses of nitrogen addition should have a similar C/N ratio, regardless of the type of nitrogen source. The bark added had a higher lignin content than stemwood, and thus, the designed addition of bark from 0 to 20% should form a gradient from a low to a high ratio of lignin to total carbohydrates in the substrates. It is understood that the range of bark doses also represents different assortments from stemwood (0% bark) to whole trees or branches with bark [17].

Table 2 Experimental design and fractions of substrate ingredients (% of DM). Bark and whey fractions are the design factors. Total carbon (C) and total nitrogen (N) were used to calculate the C/N ratio

Treatment	Ingredients					Expected content	
	Bark	Whey	Stemwood	Grain	Bran	N	C/N
N 1	0	0	80	10	10	0.51	97.0
N 2	0	1	79	10	10	0.64	75.4
N 3	0	2	78	10	10	0.78	61.5
N 4	10	0	70	10	10	0.55	90.5
N 5-1	10	1	69	10	10	0.69	71.6
N 5-2	10	1	69	10	10	0.69	71.6
N 5-3	10	1	69	10	10	0.69	71.6
N 6	10	2	68	10	10	0.83	59.0
N 7	20	0	60	10	10	0.59	85.0
N 8	20	1	59	10	10	0.73	68.2
N 9	20	2	58	10	10	0.87	56.8

2.3 Substrate preparation and shiitake cultivation

The substrates were prepared by mixing all ingredients according to Table 2; subsequently, water was added to adjust the moisture content of the substrate to 65%, which is a usual industrial practice. The pH was adjusted to around 6.5 by adding 1% $CaCO_3$ based on substrate DM.

The moisturised substrate was packed into transparent polypropylene microcontainers (125 × 65 × 80 mm) which were sealed by a lid equipped with microporous filters for gas exchange and biofiltration (Microsac, http://saco2.com/). Each container was filled with 200 g wet substrate (70 g on DM) and then pasteurised immediately in an oven at 85 °C for 4 h in the same way as in a previous study [11]. After that, the containers were left overnight in the oven to cool down to room temperature before inoculation.

Inoculation was done manually under a sterile hood. Each substrate container was inoculated with 5 g of shiitake spawn M3790 (2.5% of wet mass) (Mycelia BVBA http://www.mycelia.be/). After that, the containers were incubated under controlled conditions at around 22 °C and 70% relative humidity in the dark in a climate chamber. When the entire block was fully covered with mycelia, the colonisation period was considered complete. When the mushroom fruit bodies emerged, the plastic lid was removed, the temperature was lowered to 18 °C, humidity was increased to 90% and some light (about 500 lx) was induced in the climate chamber until the harvest was completed.

2.4 Mushroom harvest and yield

According to the current standard practice in most European mushroom industries, only one harvest (first flush) of fruit

Table 3 Substrate mass and content of major components in initial and spent substrates. All data are based on dry mass (DM)

Model components		Substrate mass		KLL		ASL		Glucan		Xylan		Extractives	
Bark %	Whey	Initial g	SMS	Initial %	SMS	Initial %	SMS	Initial %	SMS	Initial %	SMS	Initial %	SMS
0	0	70	47.5	15.3	9.2	8.3	4.7	28.8	30.9	15.2	9.7	11.8	27.3
0	1	70	48.6	15.8	9.3	8.6	5.3	28.2	33.1	14.3	8.5	11.5	24.9
0	2	70	46.5	15.2	9.6	8.5	5.1	28.7	31.5	14.9	8.5	12.6	28.3
10	0	70	47.9	18.4	10.4	8.1	4.7	27.1	29.9	16.5	8.6	11.1	28.3
10	1	70	45.2	18.2	10.6	7.4	4.5	26.0	28.8	15.2	8.8	14.3	32.3
10	2	70	46.2	17.7	10.8	7.9	4.2	25.4	25.1	14.4	8.7	13.4	34.2
20	0	70	45.3	21.0	11.6	7.3	4.2	24.4	28.0	14.7	11.4	13.6	32.5
20	1	70	47.5	21.2	12.4	7.1	5.0	24.1	27.5	13.1	11.8	12.6	28.6
20	2	70	47.2	20.9	12.7	7.4	5.0	23.6	25.7	13.5	11.2	13.5	29.1
Mean value		70	46.6	18.2	10.7	7.7	4.7	26.2	28.9	14.7	9.5	13.0	30.0
Standard deviation		–	0.8	0.1	0.1	0.2	0.2	0.2	0.6	0.2	0.2	0.6	1.3
Effects of													
Bark		–	ns	2.7a	1.5a	− 0.6b	ns	− 2.3a	− 2.4b	− 0.5c	1.3a	ns	ns
Whey		–	ns	ns	0.3c	ns	ns	− 0.4c	ns	− 0.6c	ns	ns	ns
Bark × bark		–	ns	ns	ns	ns	ns	ns	ns	− 1.2b	1.5b	ns	− 3.4a
Whey × whey		–	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Bark × whey		–	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

KLL Klason lignin, *ASL* acid-soluble lignin, *ns* non-significant ($p > 0.05$), *a* significant at $p < 0.001$, *b* significant at $p < 0.01$, *c* significant at $p < 0.05$

bodies was conducted. The fruit bodies were harvested manually and then dried at 45 °C for 96 h to determine the DM. The date of harvesting was registered for each individual container. Yield was calculated as the weight of fresh fruit bodies (90% water) divided by the DM of the initial substrate for each container and expressed as grams of fresh fruit body per kilogram of dry substrate.

2.5 Substrate sampling

Sampling was performed for initial (day 1, before pasteurisation) and spent substrates (day 65–80, immediately after mushroom harvest). Whenever sampling was carried out, the entire substrate block from each container was manually collected as one sample. The substrate samples were dried at 45 °C for 96 h, milled to ≤ 0.5 mm and stored in airtight plastic bags at room temperature prior to further analyses.

2.6 Chemical analysis

Prior to chemical analysis, the replicated samples of each treatment were pooled into a single grand sample containing equal proportions (20% by weight) of each replicate. Initial substrates and SMS were used for wet chemical analysis with two replicates.

The chemical composition of initial substrates and SMS was determined using standard procedures for wood analysis. The extractive content was determined by successive extraction with water and ethanol according to an NREL protocol [27]. The structural components were determined by analytical acid hydrolysis followed by quantitation of the sugars and lignin [28]. Glucose and xylose in the hydrolysates were analysed via HPLC (Shimadzu, Kyoto, Japan), using a Shodex NH_2P-50 4E column and an RI detector operating at 50 °C. As the mobile phase, we used HPLC-grade acetonitrile, supplied at a flow rate of 1.0 mL/min. Klason lignin was determined gravimetrically as the solid residue remaining after analytical acid hydrolysis, while for acid-soluble lignin contained in the analytical acid hydrolysate, spectrophotometric determination at 240 nm (Shimadzu, Kyoto, Japan) was used. 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, following the method described in [11].

2.7 Mass degradation of components

The mass degradation of major lignocellulose components from the initial mass could then be calculated using the following equation:

Relative mass degradation (%)

$$= [1-(MSMS*CSMS/MINI*CINI)]*100,$$

where M and C refer to mass and content of component (cellulose, hemicellulose or lignin) of SMS and initial (INI) substrates, respectively. All data are based on dry mass.

2.8 Enzymatic hydrolysis

Analytical enzymatic saccharification [29] was used for determining the enzymatic susceptibility of cellulose contained in the initial substrates and in the SMS. For each sample, 50 mg DM was suspended in 900 μL of 50 mM sodium citrate buffer (pH 5.2) in 2.0-mL Eppendorf tubes. The tubes containing the reaction mixture were placed in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland) at 45 °C and 170 rpm for 1 h for mixing and attemperation. After that, 6 μL of Cellic CTec2, an enzyme blend containing cellulases, β-glucosidases and hemicellulases, acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), was added. The final enzyme activity in the reaction mixture was 100 CMCase units per gram of biomass. After adding the enzyme blend, the tubes containing the reaction mixture were incubated for 72 h under the above-stated conditions. At the end of the hydrolysis, the tubes were centrifuged; the supernatant was stored frozen at − 18 °C until further analyses, and the precipitate was discarded. Glucose in the supernatants was analysed by HPLC and used for calculating the enzymatic convertibility of cellulose.

2.9 Statistical analysis

Multiple linear regression (MLR) was used to model the relationship between the response variables and the independent factors as well as the factor-to-factor interactions. Modelling and statistical evaluation were performed using the MODDE 11.0 software (Umetrics AB, Umeå, Sweden). The MLR models were evaluated using the coefficients of determination (R^2 and Q^2), which explained the goodness of fit and the predictive ability of the model; R^2 and Q^2 values close to 1 indicate that the model fits the data very well. For each response, a model including all the independent factors and their interactions was created. Terms showing no significant effect on the target response variable ($p > 0.05$) were excluded from the model to obtain optimised R^2 and Q^2 values, and the model was considered reliable. Principal components analysis (PCA) was used to examine the relations and relative importance of the compositional variables of SMS. The data matrix of compositional variables (11 × 7) was analysed by PCA using SIMCA 14.0 (Umetrics AB, Umeå, Sweden) after mean-centring.

3 Results and discussion

3.1 Effect of nitrogen additive sources

Although in the urea-containing substrates, the start of shiitake mycelia colonisation was evident, growth was terminated after 1–2 weeks of incubation. Survival was longer in the substrate with 0.5% addition of urea than in that with 1% addition. In all substrates with added NH_4NO_3, mycelia died within the first week. The two non-protein nitrogen sources at the given doses were apparently not preferred by the shiitake. However, shiitake mycelia grew and fructified well in the substrates supplemented with whey powder that contained 87% of protein, < 1% of carbohydrates, 1% of fat and 0.5% of fibre (data from producer). The major results reported below are therefore from the experiments with whey as a nitrogen source.

3.2 Composition of the initial substrates

The initial substrates based on the design (Table 2) contained varying concentrations of nitrogen, lignin and carbohydrates, depending on the different additions of whey and bark. Actual nitrogen content of the dry substrates ranged between 0.54 and 0.84%, while total lignin, including Klason lignin (KLL) and acid-soluble lignin (ASL), amounted to 23.6–28.3%, and carbohydrates, including glucans (GLU) and xylans (XYL), fluctuated between 37.1 and 44.0% (Table 3). Figure 1 shows the MLR models for the two most important compositional characteristics, namely the total nitrogen content and the total lignin/carbohydrates ratio, of the initial substrate. The models had very good predictive capacity for the initial substrate composition for both the nitrogen content ($R^2 = 0.98$ and $Q^2 = 0.94$) and the total lignin/carbohydrate ratio ($R^2 = 0.98$ and $Q^2 = 0.93$). The condition numbers of the models were 1.7 and 2.7, suggesting a valid experimental design [30].

As expected and indicated in Table 2 and Fig. 1, the added whey played a positive and significant ($p < 0.05$) role in actual nitrogen loading of initial substrate. Bark addition was significantly ($p < 0.05$) and positively correlated with lignin content but negatively correlated with the amount of carbohydrates. The contents of both glucan and xylan in the substrate were diluted by the addition of bark. The added whey contributed almost no carbohydrate (< 0.001% DM) to the substrate, but it had a negative influence on the content of carbohydrates due to a dilution effect.

3.3 Effects of bark and whey addition on shiitake mushroom production

The number of days from inoculation to the first harvest in this experiment, i.e. cultivation time, varied from 66 to 85 days and was significantly ($p < 0.01$) affected by both whey and bark ratio (Fig. 2a). The effect of whey was positively and

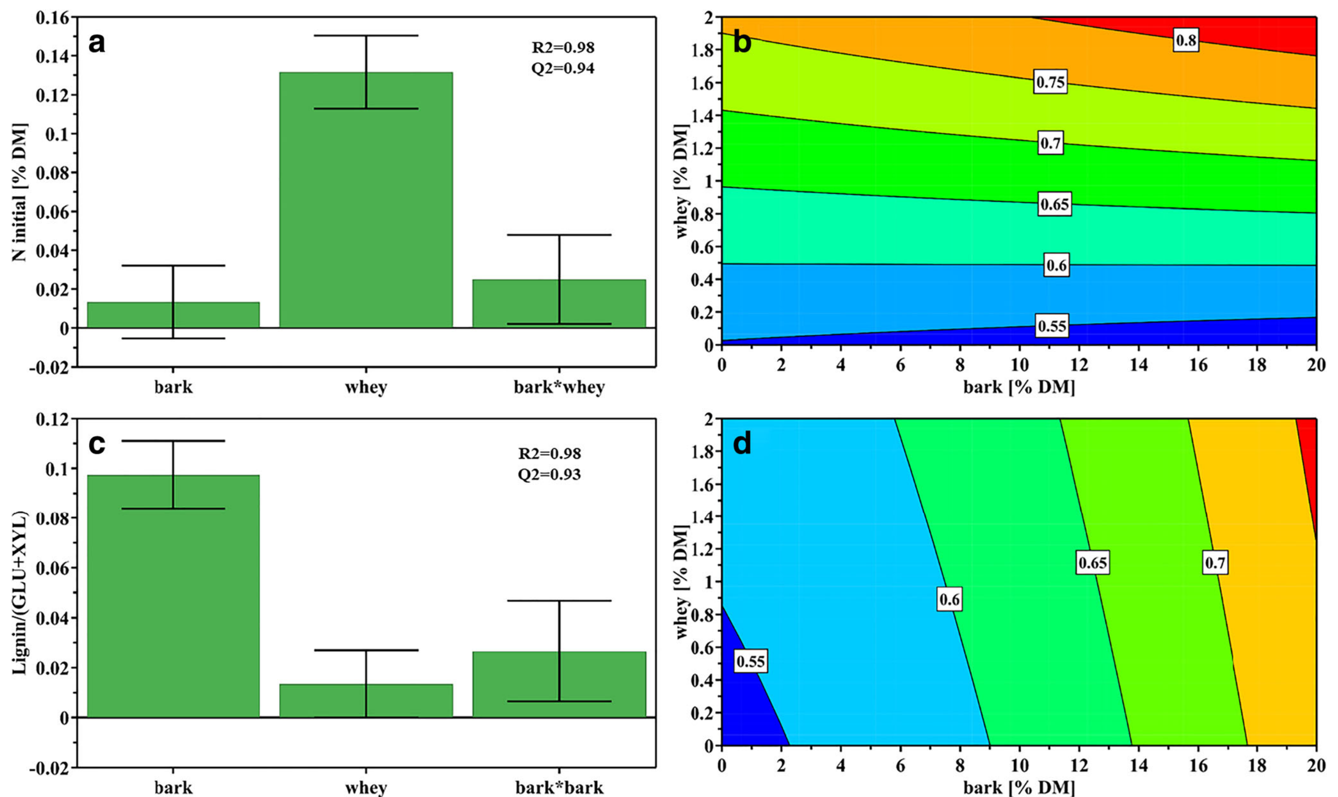

Fig. 1 Actual total nitrogen content (**a** and **b**) and ratio of lignin to carbohydrate (**c** and **d**) in initial substrate in relation to added whey and bark. **a** and **c** Main effect plots for scaled and centralised factors, the bars referring to 95% confidence level. **b** and **d** Response contour plots predicted with the MLR model

linearly correlated with the length of cultivation time, while the effect of bark showed a quadratic function with cultivation time. As indicated by the response contour plot (Fig. 2b) based on the MLR model ($R^2 = 0.78$, $Q^2 = 0.46$), the shortest cultivation period (66 days) was found for the initial substrate composed of 10.5 ± 2.5% bark, corresponding to a lignin/carbohydrate ratio of 0.60–0.65, together with < 0.2% whey addition, corresponding to a nitrogen content of 0.54–0.55% DM. Ratios higher or lower than the above-mentioned values for bark and N would slow mycelium growth and fructification resulting in longer cultivation time.

Mean shiitake mushroom yield (wet mass with 90% moisture content) reached up to 650.8 g kg^{-1} dry substrate, ranging from 472.3 to 773.6 g kg^{-1} (Fig. 2c). These values are comparable with the yield achieved in our previous report using a substrate that contained the same ingredients as in this study but without whey nor bark [11]. The MLR model for yield was not acceptable because of the low Q^2 value. There was only a marginally significant and quadratic relation with bark addition ($p = 0.04$), showing that the average fresh mushroom yield from the substrates with 10% bark (610.1 g kg^{-1}) was lower than that from the substrates with 0 and 20% bark (671.2 g kg^{-1} for both). The fast mycelial spreading with 10% bark addition shortened the cultivation time, but was not necessarily related to yields, which is in accordance with previous reports [31]. The exact underlying reasons could not be given directly by this study, but hypothetic explanations might be attributed to the physical and chemical characteristics of the bark.

It is noticeable that the shiitake yields from this study compare favourably with the values reported in other works using higher nitrogen loadings. For instance, our yields were comparable to those reported by Lin et al. [13] (yield = 650.8 g kg^{-1} dry hardwood substrate), despite higher N loadings in their study (0.8–1.4%), and even higher than those reported by Philippoussis et al. [32] (yield = 251 g kg^{-1} dry oak substrate, $N = 1.2\%$) and Ozcelik and Peksen [33] (yield = 437 g kg^{-1} dry hazelnut-husk substrate, $N = 0.8\%$). Our results are even more remarkable considering that they were obtained from only one harvest, whereas in the above-referred reports, the yields were mostly obtained from two to three harvests. Based on this, it can be concluded that the designed N levels used in this study are good enough to reach a profitable mushroom yield, when whey is used as the N-additive to birch-based substrate.

3.4 Compositional changes of substrates during shiitake cultivation

Compositional changes can be typically characterised by the changes in mass of the major lignocellulosic components. Based on the substrate mass remaining and the component

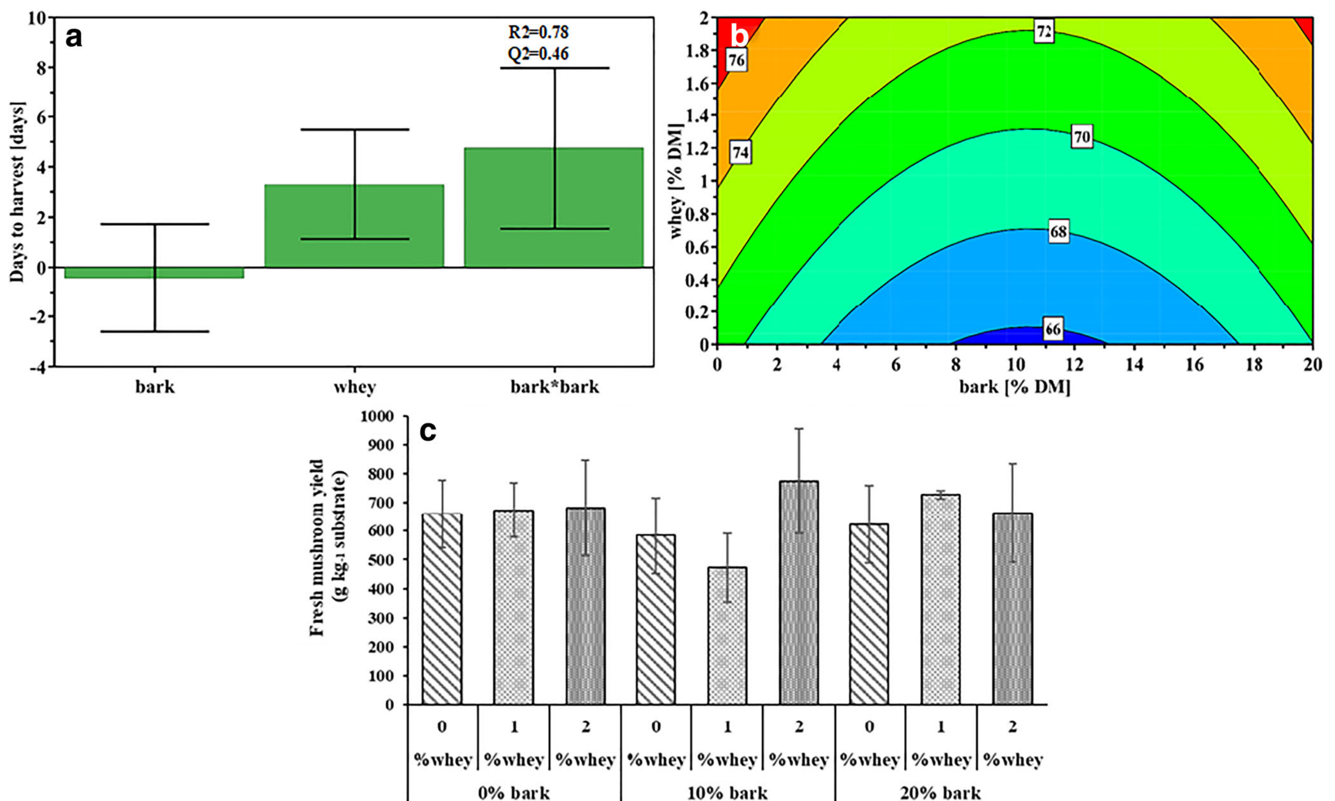

Fig. 2 Cultivation time (days from inoculation to harvest) (**a** and **b**) and fresh mushroom yield (**c**) in relation to additions of bark and whey. **a** Main effect plot for scaled and centralised factors, the bars referring to 95% confidence level. **b** Response contour plot predicted with the MLR model

content in SMS (Table 3), the relative change in mass of each component was calculated using the equation described in Section 2.7.

The average relative reduction in mass of the major components from the initial levels showed the following order: KLL (60.8%) > ASL (59.5%) > XYL (56.7%) > GLU (26%). The reductions in KLL and XYL mass were closely correlated with the amount of added whey and bark and could be well described by the MLR predictive models ($R^2 = 0.83$ and $Q^2 = 0.40$ for KLL; $R^2 = 0.89$ and $Q^2 = 0.65$ for XYL), as illustrated in Fig. 3. The MLR models for ASL and GLU were not acceptable ($Q^2 < 0$), and their mass degradation was considered comparable between treatments. No models were achieved for the relative changes in mass of extractives either; there were no significant differences in extractives between treatments.

As shown in Fig. 3a, b, the degradation ratio, i.e. relative change in mass, of KLL increased ($p < 0.05$) with an increase of bark but decreased with increasing whey fraction. The lowest degradation was 58.3% when adding 0–1.1% bark and 0.8–2% whey and when adding 19.3–20% bark and 1.9–2% whey, where initial nitrogen levels corresponded to > 0.62% and a lignin/carbohydrate ratio of 0.54–0.56% and 0.74–0.75%. The highest ratio was about 63.5% with the addition of 0–0.07% whey and 14–19% bark, corresponding to N loading at 0.53–0.54% and a lignin/carbohydrate ratio of around 0.66–0.71.

Mass degradation of xylan was marginally affected by whey addition, but had a significant and strong quadratic correlation ($p < 0.05$) with bark ratio (Fig. 3c). The MLR model contour plots (Fig. 3d) indicate that the lowest xylan degradation region was found (44%) for the addition of about 20% bark and 1.5–2% whey, where nitrogen levels corresponded to > 0.75% and the lignin/carbohydrate ratio was around 0.75. The highest degradation was about 64%, when additions of whey and bark were 0–0.85% and 4.1–9.1%, respectively, which corresponded to an N content of 0.55–0.64% and a lignin/carbohydrate ratio of 0.57–0.62.

Compared with those of Klason lignin and xylan, mass reduction of glucan was remarkably lower: around 18.5–34.9% of glucan in the initial substrate was consumed during cultivation. Glucan mass degradation was significantly affected by bark only ($p = 0.04$) and tended to be slightly higher at 10% bark than at 0 and 20% (Fig. 4a, b).

Bark addition was significantly correlated with the mass reduction patterns of all three components, namely lignin, xylan and glucan (Figs. 3 and 4), while whey played a significant role in Klason lignin degradation only. This fact suggests that, within the conditions investigated in this study, the addition of bark was more important for lignocellulose degradation than that of whey. The amount of added bark resulted in a gradient of lignin/carbohydrate ratios (Fig. 1), and it was

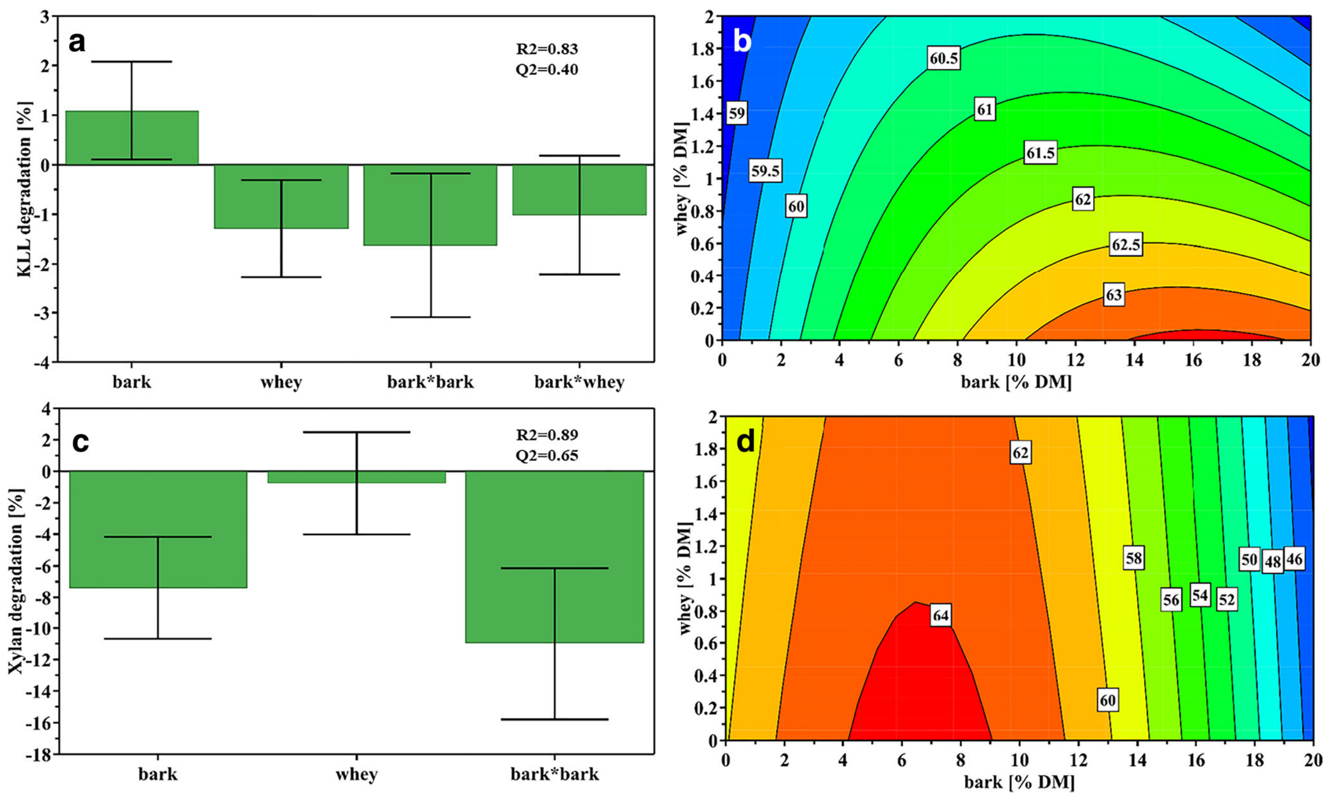

Fig. 3 Effects of whey and bark on the mass degradation of Klason lignin (**a** and **b**) and xylan (**c** and **d**). **a** and **c** Main effect plots for scaled and centralised factors, the bars referring to 95% confidence level. **b** and **d** Response contour plots predicted with the MLR model

possible to identify a threshold (around 0.65) distinguishing favourable ratios for lignin and xylan degradation. Lignin/carbohydrate ratios above 0.65 were favourable for lignin degradation, while values below 0.65 favoured xylan degradation. It is interesting that with the increase of the bark fraction and the lignin/carbohydrate ratio, the shiitake mycelia shifted their preference from degrading xylan to degrading lignin. In this aspect, the addition of bark functioned as a regulatory factor.

The added bark might also have changed the physical structure of the substrate. Since birch bark was fluffier and had a lower bulk density than the stem sawdust (Table 1), its addition could have increased the porosity and aerobic microenvironment inside the substrate. Elevated oxygen levels would increase the rate of lignin degradation through the promotion of peroxidase enzyme secretion [34, 35]. However, the resistance of bark lignin as compared to stem lignin might be attributed in part to the inhibiting effect of tannins and suberin on fungal growth [19], which could be why fructification was delayed when the bark ratio was close to 20% (Fig. 2b), resulting in impairment of xylan degradation with high bark loadings (Fig. 3d). It is remarkable that lignin (Fig. 3b) and

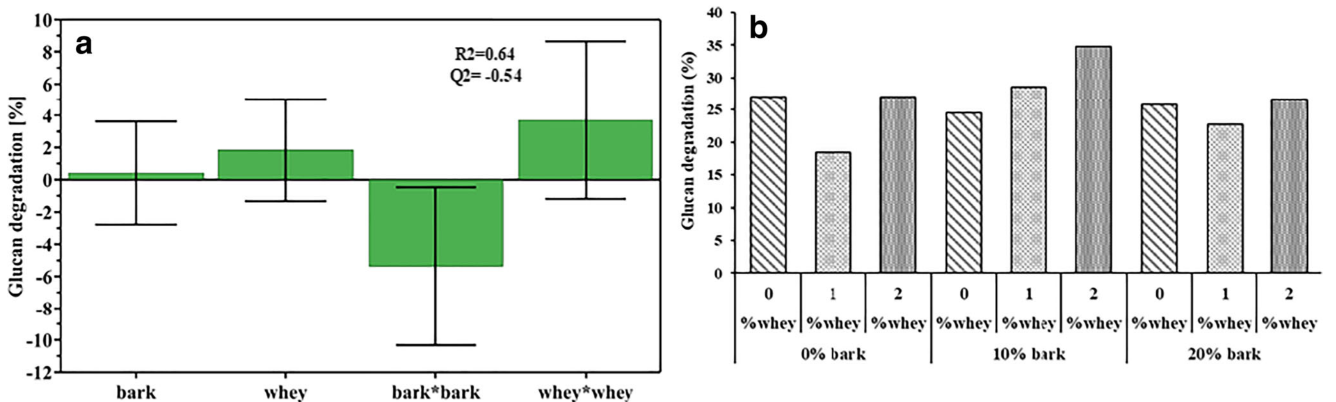

Fig. 4 Effects of whey and bark on glucan mass degradation. **a** Main effect plot for scaled and centralised factors, the bars referring to 95% confidence level. **b** Mass change ratio of components in the initial substrate to those in spent substrate

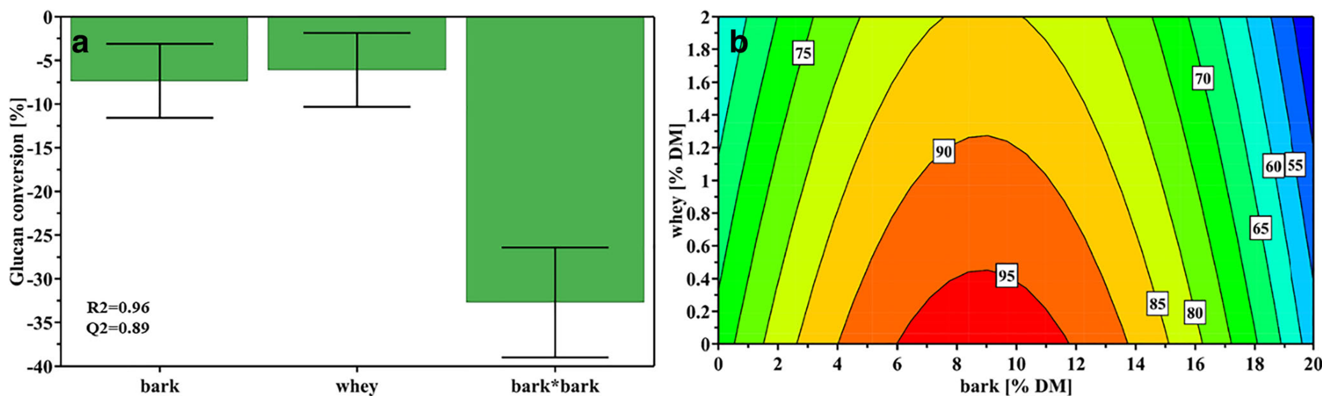

Fig. 5 Glucan conversion (**a** and **b**) during enzymatic hydrolysis of SMS. **a** Main effect plot for scaled and centralised factors, the bars referring to 95% confidence level. **b** Response contour plot predicted with the MLR model

glucan (Fig. 4) degradation was not affected by high doses of bark as much as xylan degradation was.

From contour plots paired with responses (Figs. 2 and 3), a nitrogen content of around 0.55%, at which the highest degradation rate of Klason lignin and the shortest cultivation time were found, appeared to be optimal. The fact that Klason lignin degradation was correlated with the amount of nitrogen in the substrate is in accordance with the finding that nitrogen-limited conditions enhance the production of lignin-degrading enzymes (LiP and MnP) and thus induce the depolymerisation of lignin, facilitating colonisation [24, 25]. In this study, the resulting lignin mass degradation (59–64%) was higher but the cellulose mass degradation was lower (19–35%) than those reported for shiitake cultivation on other hardwood materials [13, 36]. Lin et al. [13] reported an average lignin mass degradation of 54% and a cellulose mass degradation of 69% after shiitake cultivation, with no significant differences between nitrogen loadings of 0.8–1.4%. Based on data of Atila's [36] study, a calculation was performed and showed that up to 45% of lignin mass degradation and 46% of cellulose mass degradation resulted from oak substrate with 0.34% nitrogen content. Interestingly, the N loadings of 0.51–0.87% in the initial substrate in this study were moderate, but seemed to have resulted in a more selective degradation of lignin and cellulose, which could be a positive feature for a biological pretreatment of birch-based substrate. However, future studies are needed to examine whether N loadings may interact with substrate species to affect the biological pretreatment process during shiitake cultivation.

3.5 Enzymatic digestibility of substrates

As indicated in Fig. 5, the enzymatic digestibility of glucan contained in the SMS ranged from 41.3 to 92.8%, compared with 20–22% for raw/initial substrate prior to cultivation [11], showing a considerably positive effect of fungal pretreatment. Enzymatic digestibility showed a negative linear correlation with whey ($p < 0.05$) and a strong quadratic correlation with bark addition ($p < 0.001$). Only around 50% of glucan could be converted to glucose when bark addition was closer to 20% and whey addition was > 1.3%, which could be explained by less degradation of lignin and xylan (Fig. 3) and probably also by the high ratio of intermediate products from lignin degradation, such as organic acids, which can inhibit hydrolytic enzymes [37]. The relatively lower digestibility at a low bark content might be a consequence of the low degradation of

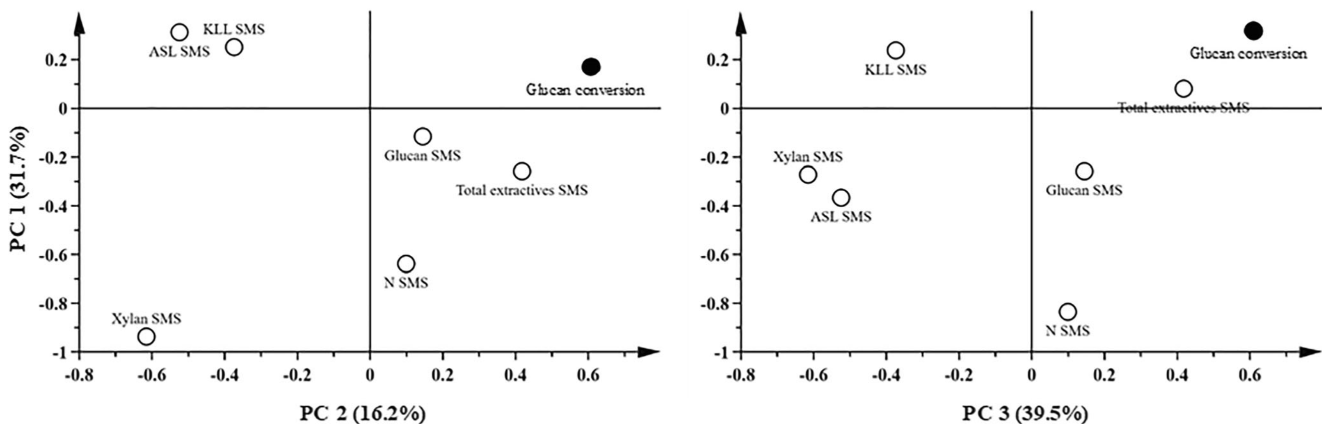

Fig. 6 PCA loading plot showing major chemical components of SMS (open circles) and glucan conversion (filled circles) following enzymatic hydrolysis

lignin during fungal growth under those conditions (Fig. 3b). Initial nitrogen loading below 0.59% (i.e. 0.45% whey) and a lignin/carbohydrate ratio from 0.59 to 0.64 (i.e. 8.9 ± 2.9% bark) resulted in the highest hydrolytical conversion (95%) of glucan contained in the SMS, which remarkably coincided with the most favourable conditions, in terms of nitrogen content and lignin/carbohydrate ratio in the substrate, for the degradation of both lignin and xylan (Fig. 3).

Based on the data matrix (11 × 7) of major chemical composition and enzymatic digestibility of glucan in SMS, a PCA was performed. The three first PCA components explained 87.4% of the total variation. The loading plots, for both PC1 × PC2 and PC1 × PC3 (Fig. 6), show that lignin and xylan are on the opposite side to glucan conversion, which is grouped together with total extractives and glucan content, confirming a negative effect of lignin and xylan on enzymatic hydrolysis [4] and a positive relation between glucan conversion and glucan concentration in SMS. It is understandable that, because of the lower lignin and xylan contents (Table 3), the spent substrate remaining after shiitake cultivation is less recalcitrant than the initial substrate, and therefore, glucan is more digestible by the enzymes (see also [11]). The increase in extractives was an effect of major biochemical processes. Theoretically, the incomplete degradation of cellulose might result in short-chained polysaccharides and in oligosaccharides remaining in the SMS [38]. In the presence of cellulases, those oligo- and short-chained polysaccharides undergo hydrolysis, which could explain why the high content of extractives in the SMS is positively related to glucan conversion.

4 Conclusions

This study showed that by regulating the addition of whey/nitrogen and bark in the initial substrate, it is possible to minimise glucan degradation and maximise the degradation of lignin and hemicellulose during shiitake cultivation, thereby enhancing enzymatic saccharification of cellulose in the spent substrate. Keeping in mind that whey and non-debarked birch wood are underused bioresources, our results shall have a considerable implication to promote a cost-effective, energy-efficient and environmentally friendly combined production of food and renewable energy by using forest residues, which would finally benefit a biobased circular economy.

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Authors' contributions SJX initiated the project and designed the experiment. FC co-designed the experiment, performed fungal cultivation and chemical analyses and drafted the manuscript. CM provided the methods for chemical analysis. SJX, CM and MF provided key comments for manuscript writing. All authors read and approved the final manuscript.

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Code availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication All authors agree to the publishing of the paper.

Abbreviations SMS, spent mushroom substrate; DM, dry mass; KLL, Klason lignin; ASL, acid-soluble lignin; GLU, glucan; XYL, xylan; MLR, multiple linear regression; CCF, central composite face; PCA, principal component analysis

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