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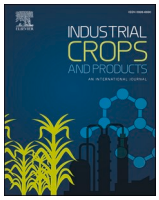

Investigation of crop straw for edible and medicinal fungi cultivation: Assessment of lignocellulose preprocessing and spent substrate biofuel properties

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ABSTRACT

Pretreatment with white-rot fungi has advantages of low inputs of energy and chemicals for reducing the recalcitrance of woody biomass for cellulosic ethanol production. This study investigated the effects of substrates ranging from wood to wheat straw on edible and medicinal fungi production, lignocellulose degradation, cellulose saccharification and ethanolic fermentation of the produced hydrolysates. Shiitake cultivation resulted in the most substantial degradation of lignin and xylan. Reishi produced a selective degradation pattern in terms of preferential xylan removal. Oyster had poor performance in lignocellulose degradation. Shiitake and reishi had high reactivity of S-lignin. The strong recalcitrance of >10 % wheat straw addition for mushroom cultivation might be attributed to the low S:G ratio of the substrates. Compared with the substrate comprising a single hardwood, 10 % wheat straw addition optimised the integration process, resulting in a generally comparable fruiting body yield and higher lignocellulose degradation. The shiitake-based and reishi-based spent mushroom substrates (SMSs) contained ~21 % glucan, which released 84.4 % and 33.5 % of potentially achievable glucose upon enzymatic saccharification, respectively. The SMS hydrolysates ensured ethanol yields corresponding to 78.0 %–83.2 % of the theoretical value in fermentation. The lignocellulose degradation–derived by-products following the fungal pretreatment showed a notable difference compared with thermochemical methods and might cause inhibitory effects on yeast. This study provides valuable insights into the cause of crop straws's inhibition of white-rot fungi production and reveals the potential of fungal pretreatment as a biorefinery approach producing food and biofuel.

1. Introduction

Lignocellulosic biomass is currently the most essential renewable source available on a large scale in the world for producing cellulosic ethanol. However, owing to feedstock recalcitrance, enzymatic saccharification of raw lignocellulose results in low rates and yield. Pretreatment is required to remove hemicellulose and/or lignin to improve the enzymatic hydrolysis of cellulose (Jönsson and Martín, 2016; Mankar et al., 2021). Biological pretreatment using lignin-degrading microorganisms, such as edible and medicinal white-rot fungi, is also an interesting strategy.

In previous studies, shiitake (*Lentinula edodes*) cultivation on hardwood-based substrates resulted in 75 % and 70 % mass degradation of lignin and hemicelluloses, respectively (Chen et al., 2022b; Xiong et al., 2019). Owing to its lower recalcitrance, cellulose contained in spent mushroom substrate (SMS) has higher susceptibility to enzymatic saccharification (up to 88 %), which corresponds to an increase of 4–5 times compared with raw wood (Chen et al., 2022c; Xiong et al., 2019). The integrated production of biofuel and food from lignocellulosic residues suits the biobased circular economy strategy. Pointing in that direction, the overall objective of this study was to tackle relevant scientific issues regarding biological pretreatment using edible and medicinal white-rot fungi.

Considerable studies have reported good shiitake production in substrates with hardwood sawdust as a basic ingredient (Royse et al., 2017). Agricultural residues, such as wheat and rice straw, are difficult

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to colonise and are rarely used for fruiting body production. However, from the perspective of developing countries, such as China, where agricultural residues are large, concentrated and underutilised (Fang et al., 2019), it is desirable to extend the mushroom substrate range from wood to indigenous agricultural residues on an industrial scale for both edible mushroom and biofuel production.

Woody and agricultural residues differ in chemical and structural features. For example, agricultural biomass typically has lower lignin and carbohydrate contents than woody biomass (Shirkavand et al., 2016). Additionally, there is an obvious difference in the lignin structure between biomass. Syringyl (S) and guaiacyl (G) are the main units of the lignin macromolecule. Hardwood birch, alder and aspen have S:G ratios of 2.4–3.8, whereas wheat bran lignin consists of 66.6 % G units (Chen et al., 2022b; Wang et al., 2018). The first scientific issue in this study was the inclusion of wheat straw in the substrate formulation for white-rot fungi cultivation and arriving at a comprehensive view of the main causes of wheat straw's inhibition using different analytical techniques considering both chemical composition and structure.

The second issue was the assessment of edible and medicinal white-rot fungi species in lignocellulose pretreatment. Oyster (*Pleurotus ostreatus*) is the second most cultivated edible white-rot fungi worldwide after shiitake, and global production accounts for approximately 19 % of the total cultivated edible mushrooms (Royse et al., 2017). Oyster mushrooms are aggressive colonisers of a wide array of substrates (Fernandes et al., 2015), and a previous study has reported that they can achieve complete mycelium growth and good fruitification on softwood (Chen et al., 2020). This suggests a great potential to evaluate oyster mushroom as a model species for agricultural residue processing. *Ganoderma lucidum*, known as reishi, is a traditional medicinal mushroom and has been extensively cultivated and used in Asian countries because of its valuable pharmacological activities, such as hyperglycemic, immunomodulatory and anti-tumour activities (Wu et al., 2024). However, there is little, if any, published research regarding the capacity of oyster and reishi in lignocellulose preprocessing to produce cellulosic ethanol.

Thermochemical pretreatment is currently a method of high interest for industrial implementation, but it often involves side reactions that result in lignocellulose degradation–derived by-products, such as furan aldehydes, aliphatic carboxylic acids and phenolic compounds, exerting toxic effects on ethanolic fermentation (Jönsson and Martín, 2016). In a previous shiitake study, aliphatic carboxylic acids, including acetic acid, formic acid and levulinic acid, were detected in low amounts in the SMS (Chen et al., 2022b). Furan aldehydes and 5-hydroxymethylfurfural were found to be negligible during shiitake cultivation, and the fraction of phenolic compounds in the SMS ranged from 1.8 % to 2.4 %, corresponding to a 1.4–7.2 fold increase (Chen et al., 2022b). Investigating whether the degree of fungal degradation affects by-product accumulation and the potential toxicity of by-products on ethanolic fermentation is another issue of this study.

The objective of this study was to experimentally investigate the potential of using wheat straw for the cultivation of shiitake, oyster and reishi using hardwood chips as a reference. This study examined (1) the regulatory roles of wheat straw in the substrate on white-rot fungi production and lignocellulose pretreatment, and the main causes of wheat straw's inhibition, (2) the potential of different edible and medicinal white-rot fungi species in lignocellulose pretreatment, (3) the cellulose enzymatic saccharification from the SMSs and (4) the accumulation of lignocellulose-derived by-products in the resulting hydrolysates and their potential toxicity on ethanolic fermentation.

2. Materials and methods

2.1. Fungal strains

Spawns of commercial shiitake (Shenxiang 16), oyster (Xiafu 1) and reishi (Hong) mushrooms provided by the East Anhui Experimental Station of Anhui Agricultural University (Mingguang City, Anhui Province) were used in this study. The isolates were maintained on potato dextrose agar at 4°C in the dark before use. Spawns were then prepared by cultivating isolates on substrates composed of 78 % hardwood mixtures, 20 % wheat bran and 2 % gypsum at 23°C in the dark, running to allow substrates to become fully colonised by the selected strain.

2.2. Materials and mushroom substrate preparation

Hardwood mixtures with a particle size of 5–10 mm were supplied from a mushroom grower located in Hubei province, China. Wheat (*Triticum aestivum L.*) straw was collected and dried immediately after harvest in Mingguang City and ground to <1 cm before use. Wheat bran (<1 mm) was purchased from a local agriculture cooperative.

The substrate ingredients were mixed according to the proportions indicated in Table 1. The pH of the substrates was adjusted to approximately 6.4 by adding $CaCO_3$ on 1 % of dry mass (DM). All substrates were rewetted to moisture contents of 54 %–57 % using distilled water. After blending all ingredients, the moisturised substrates (150 g on wet mass) were placed in transparent bags. Each treatment contained 10 replicates for sampling at different periods of cultivation.

Substrate pasteurisation was performed based on an autoclave using pressurised steam (121°C and 2 bar for 3 h) to remove competitive microbes. Subsequently, the substrate containers were left overnight to cool down to room temperature before inoculation.

2.3. Inoculation, cultivation and sampling

Inoculation was performed under a sterile hood. Each substrate container was inoculated with 4 g of spawn (2.7 % of wet mass). Subsequently, the substrates were incubated under controlled conditions at approximately 22°C 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 fruiting bodies emerged, the bag was removed, temperature was lowered to 18°C, humidity was increased to 90 % and some light (<300 lx) was introduced in the climate chamber until harvest was completed (only one harvest of fruiting bodies was conducted). The mushrooms were harvested manually and air-dried at 45°C. Yield was defined as the fresh weight (g) per kg dry raw substrate, where the fresh weight was normalised to a 90 % moisture content.

Samples were taken periodically at different periods of mushroom cultivation: (1) raw substrate samples were collected before pasteurisation, (2) three replicated substrate blocks were randomly collected at the colonisation stage, (3) three replicated substrate blocks were randomly collected 2 weeks after colonisation (in-between stage) and (4) the remaining four replicated substrates were collected after mushroom harvest (SMS). The abovementioned substrate samples were collected from the entire substrate container, dried at 45°C and milled to ≤0.5 mm.

Table 1
Substrate ingredients and experimental design.

White-rot fungi	Substrate ingredients (% DM)			
	Hardwood chips	Wheat straw	Wheat bran	$CaCO_3$
Shiitake/Oyster/ Reishi	80	0	20	1
	70	10	20	1
	60	20	20	1

Sampling: Raw (Day 0), Colonisation, In-between (2 weeks after colonisation), SMS

2.4. Enzymatic saccharification

2.4.1. Analytical enzymatic saccharification

The susceptibility of the raw substrates and SMSs to enzymatic hydrolysis was determined using analytical enzymatic saccharification (Gandla et al., 2018) with some modifications. For each sample, 0.5 g of DM was suspended in 5 mL of 50 mM sodium citrate buffer (pH 5.2) in 10-mL tubes. Subsequently, commercial enzyme preparation Cellic CTec2 (initial enzyme activity: 340 CMCase units/mL), which is a blend of cellulases, β-glucosidases and hemicellulases acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), was added at a load of 100 CMCase units/g biomass, and the mixture was incubated for 72 h at 50°C and 130 rpm in a horizontal circle in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland). After hydrolysis, the hydrolysate was separated by centrifugation at 10,000 rpm for 15 min to remove small particles and stored at −20°C for compositional analysis. Each experimental treatment was performed in triplicate, and the mean values and standard error (SE) were reported.

2.4.2. Preparative enzymatic saccharification

The preparative enzymatic saccharification of the shiitake-based and reishi-based SMSs (10 % wheat straw addition) was performed to produce hydrolysates for use in the fermentation experiment, as previously described (Chen et al., 2022c) with some modifications. The process is the same as analytical enzymatic saccharification, except that 20 g of the sample was suspended in sodium citrate buffer at 10 % solid content in 500-mL Erlenmeyer flasks. After hydrolysis and centrifugation, the hydrolysate was adjusted to pH 5.5 using NaOH solution and filter-sterilised through a 0.22 μm sterile filtration unit under vacuum.

2.5. Fermentation of hydrolysates

2.5.1. Inoculum and media

To prepare the inoculum, freeze-dried yeast (*S. cerevisiae* YSC2, Sigma-Aldrich, Trading Co. Ltd, Shanghai) was suspended in sterile deionised water at 35°C for 30 min. The cell concentration in each fermentation flask was 1 g/L. The nutrient solution, corresponding to a total nitrogen content of 30.9 g/L, contained 150 g/L yeast extract, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4{\cdot}7\ H_2O$ and 238.2 g/L $NaH_2PO_4{\cdot}H_2O$, as previously described (Chen et al., 2022c; Martín et al., 2018). The fresh yeast suspension and nutrient solution were prepared right before inoculation. The fermentation media consisted of 92.4 % (v/v) filter-sterilised SMS hydrolysate, 5.6 % (v/v) yeast inoculum and 2 % (v/v) nutrient solution.

2.5.2. Fermentation

Filter-sterilised SMS hydrolysates were aseptically mixed with the nutrient solution and yeast inoculum in 100-mL Erlenmeyer flasks at a working volume of 50 mL. The flasks were sealed with cotton plugs to allow the release of CO_2 formed during fermentation. The fermentation media were incubated in an Ecotron orbital incubator at 35°C and 180 rpm. Samples were taken at the beginning of the fermentation and after 2, 4, 6 and 8 h. Ethanol yield was calculated as the maximum amount of ethanol formed per 100 g of initial glucose. Each experimental treatment was conducted in quadruplicate. Mean values and standard error (SE) were reported.

2.6. Chemical analysis

2.6.1. Compositional analysis of substrates, hydrolysates and fermentation samples

The determination of the extractives and structural components in the substrates was performed using NREL methods (Sluiter et al., 2008, 2005). Analytical acid hydrolysis (AAH) was performed to determine the lignin and carbohydrate contents. 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 AAH hydrolysates were analysed with high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column and a RI detector. Elution was performed with an isocratic flow of 5 mM aqueous sulphuric acid solution. The flow rate was 0.6 mL/min, and the column temperature was set to 55°C.

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

Relative mass degradation % = $[1 - (M_{SMS} * C_{SMS})/(M_{raw} * C_{raw})] * 100$,

where M and C refer to the substrate mass and component content (glucan, xylan or lignin), respectively, of the SMS and raw substrate.

The concentrations of glucose, levulinic acid, formic acid, acetic acid and ethanol in the enzymatic hydrolysates and fermentation samples were determined by HPLC. Total phenolic compounds were quantified colorimetrically using the Folin–Ciocalteu's method with vanillin as the calibration standard.

2.6.2. 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 the collected substrates. The analysis was performed at the Plant Cell Wall and Carbohydrate Analytical Facility of the Umeå Plant Science Centre (UPSC) (Umeå, Sweden) according to the method described by Gerber et al. (2012).

2.7. Data analysis

One-way analysis of variance, followed by post hoc multiple comparisons (Duncan), was conducted to analyse the difference between data using SPSS statistical analysis software (IBM SPSS version 26.0). Differences were considered significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$. Principal component analysis (PCA) was performed to gain an overview of the data using SIMCA 14.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden).

3. Results and discussion

3.1. Fungal colonisation and fresh mushroom production

After inoculation with spawns, colonisation signals were evident in all formulated substrates composed of either a single hardwood ingredient or hardwood–wheat straw mixtures. Full colonisation required 22–30 days for shiitake (Fig. 1a), and a faster ($p > 0.05$) colonisation was observed in substrates with a single hardwood ingredient. A similar pattern was observed for oyster ($p < 0.05$). The longest colonisation time (16–16.5 days) was found for the raw substrate composed of 10 % and 20 % wheat straw. By contrast, wheat straw addition accelerated the growth of reishi mycelium. Colonisation time was shortened ($p < 0.05$) to 15 days when 20 % wheat straw was added.

Shiitake fruiting bodies were harvested 154–186 days after inoculation (Fig. 1b), and fructification was delayed with 10 % wheat straw addition. The substrate with 20 % wheat straw addition suffered from the termination of fructification. Although primordia started to appear at approximately day 170 (data not shown), they showed a considerably low number (< 3 per substrate block) and did not form fruiting bodies (observation ended on day 210). The entire time to harvest varied from 39.2 to 47 days for the oyster mushroom. There were no statistically significant differences ($p > 0.05$) between substrates. The reishi fruiting bodies cultivated in substrates composed of 10 % and 20 % wheat straw were harvested on approximately day 104, which was three weeks shorter than that with a single hardwood substrate.

The yield of fresh shiitake fruiting bodies (normalised to a moisture content of 90 %) ranged between 306 and 329 g per kg of dry substrate (Fig. 1c), with slight differences between substrates. The oyster

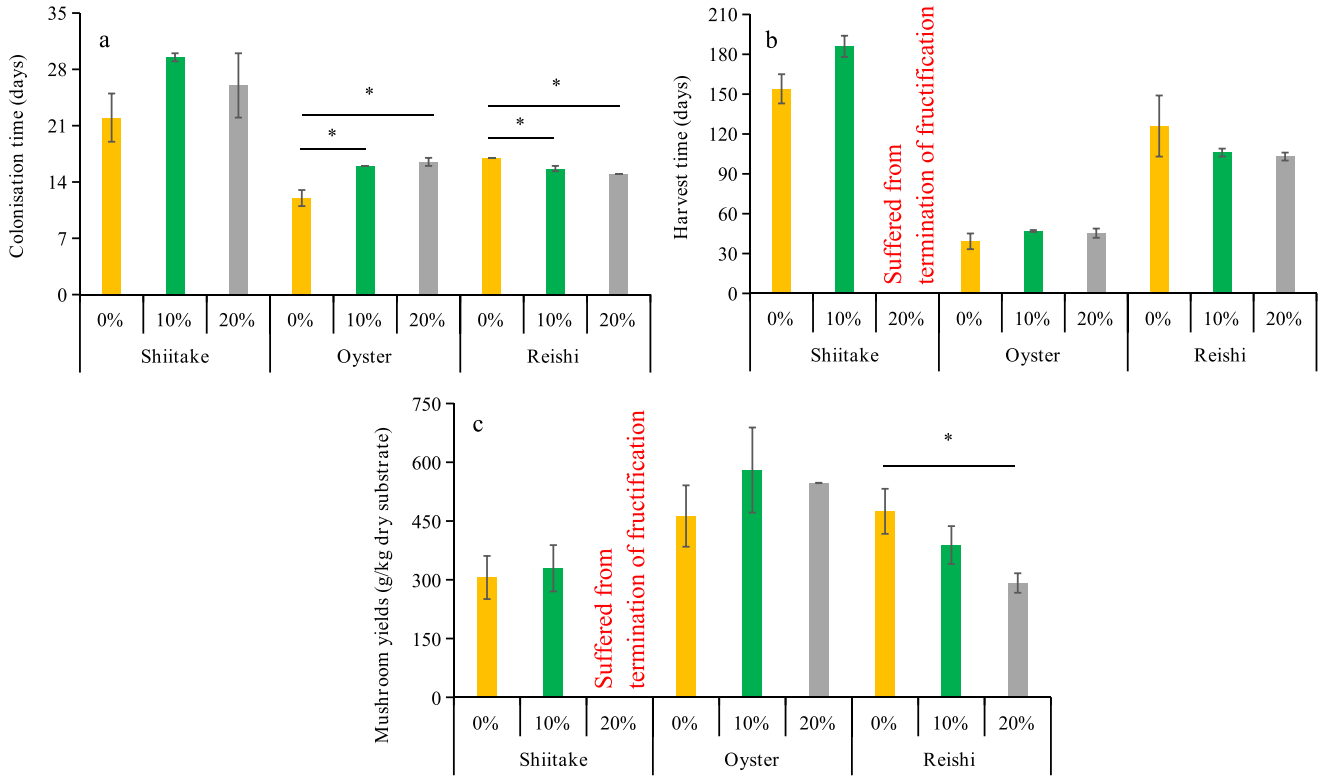

Fig. 1. Mycelium colonisation time (a), mushroom harvest time (b) and fresh mushroom yield (c) in relation to wheat straw addition (mean ± SE). * indicates significant differences ($p < 0.05$) between treatments.

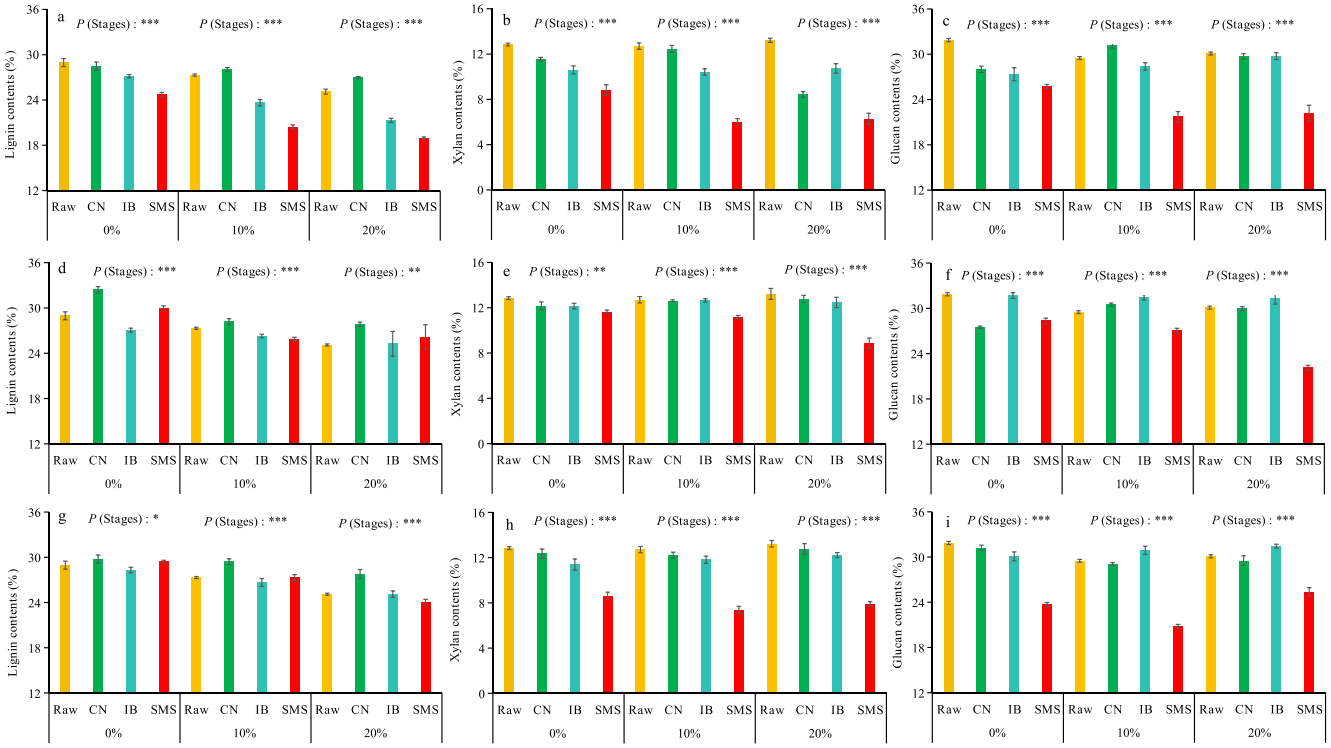

Fig. 2. Dynamics of lignin, xylan and glucan contents in the substrates over different cultivation stages of shiitake (a–c), oyster (d–f) and reishi (g–i). Data refer to mean values ± standard error (SE). *, ** and *** indicate significant differences $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. CN: colonisation, IB: in-between.

mushroom yield in the single hardwood substrate averaged 463 g per kg of dry substrate, and wheat straw addition increased the yields to 547–580 g per kg of dry substrate ($p > 0.05$). Reishi mushroom yield reached up to 385 g per kg of dry substrate, ranging from 292 to 475 g kg^{-1}. A negative correlation ($p < 0.05$) was observed between yields and wheat straw addition.

Wheat straw addition showed significant effects on mycelium growth and fructification, and the responses were variable for different white-rot fungi species. Shiitake was sensitive to the substrate ingredients, which agrees with our previous study showing that added bark (0 %–20 %) had a quadratic correlation with mushroom production and harvest time (Chen et al., 2022a). Differences in chemical features between woody and agricultural biomass are larger than divergences between stemwood and bark from the same tree species. Wheat straw addition delayed or completely inhibited the shiitake fruiting body harvest (Fig. 1). In any case, compared with a single hardwood substrate, 10 % wheat straw addition resulted in comparable fruiting body yield, which can be considered to partially substitute forest resources. Oyster mushrooms are aggressive colonisers of a wide array of lignocellulosic biomass. Softwood and cellulose fibre rejects can be used to produce substrates (Chen et al., 2020; Grimm et al., 2021). Although wheat straw addition resulted in slower mycelium colonisation and fructification, the higher yield implies a great potential of wheat straw for oyster mushroom production (Fig. 1). The fast mycelial spread with wheat straw addition shortened the reishi cultivation time but was not necessarily related to yields (Fig. 1).

3.2. Major changes in substrate lignocellulose

3.2.1. Lignocellulose composition

Woody and agricultural biomass showed considerable differences in chemical composition (Fig. 2). The lignin (sum of Klason lignin and acid-soluble lignin) and glucan contents in raw substrates ranged from 29.0 % to 25.1 % and from 31.9 % to 29.5 %, respectively. Wheat straw addition decreased the lignin and glucan contents in the raw substrates. Wood sawdust and wheat straw showed comparable levels in xylan, with a content of 12.7 %–13.2 % found in the raw substrates.

Although the substrate with 20 % wheat straw addition was not unfavourable to shiitake cultivation, the SMS was collected on day 210 for chemical analysis. The major changes in the lignin content of shiitake-based substrates, regardless of wheat straw addition, occurred after mycelium colonisation (Fig. 2a). The lignin content in the substrates showed no considerable differences between the raw and colonisation stages and decreased with time from 27.8 % to 21.4 %, on average, in SMSs. The lignin content in the substrates with 10 % wheat addition had the largest decrease, from 27.3 % in the raw substrate to as low as 20.4 % by the end of cultivation. Shiitake mushroom cultivation led to a 45.5 % reduction, on average, in the xylan content in the substrates (Fig. 2b). Although a comparable xylan content was found in the raw substrates, the substrate types had a significant effect on the content in the SMS (6.0 %–8.8 %) and 10 % wheat straw addition resulted in the largest decrease in all of them ($p < 0.05$). The major decreases in substrate glucan occurred in the middle and latter stages (from in-between to SMS) of shiitake cultivation (Fig. 2c). After shiitake mushroom cultivation, the lowest glucan content of 21.7 % was found in the SMS with 10 % wheat straw addition.

Oyster mushroom cultivation caused minor effects on lignin content (Fig. 2d). The lignin content showed a slight increase in the colonisation stage and then decreased to 25.9 %–30.0 % in SMS, which was generally comparable to the values of raw substrates. There were points of similarity in the carbohydrate dynamics (Fig. 2e and f). The xylan and glucan contents in the substrates remained comparable over time between the raw and in-between stages and showed a clear decrease at the end of the cultivation period, especially for the substrate with 20 % wheat straw addition. The xylan and glucan contents in the SMSs ranged from 8.9 % to 11.6 % and from 22.1 % to 28.3 %, respectively, and showed significant negative correlations ($P < 0.05$) with wheat straw addition.

The lignin content in the reishi-based substrates, ranging from 24.0 % to 29.8 %, varied over the growing season (Fig. 2g). The lignin contents of SMSs composed of 0 % and 10 % wheat straw were comparable to those of the raw substrates, except for the substrate with 20 % wheat straw addition showing a slight decrease at the end of the cultivation period. The carbohydrate contents in the substrates showed no considerable differences between the raw and in-between stages and showed a clear decrease in the SMSs (Fig. 2h and i). The xylan and glucan contents in the SMS were 8.0 % and 23.3 %, on average, respectively, which corresponded to reductions of 38.3 % and 23.6 %, respectively, compared with the raw substrates. Wheat straw addition had a significant ($p < 0.05$) quadratic correlation with the xylan and glucan contents in the SMS. The lowest contents (7.4 % and 20.8 %, respectively) were found with 10 % wheat straw addition.

3.2.2. Lignocellulose mass degradation

After fruiting body harvest, the average remaining mass of the whole substrate block (SMS recovery) showed the following order: shiitake (54.5 %) < reishi (62.2 %) < oyster (76.6 %) (Fig. 3). The substrate mass that remained or that was lost was associated with the fungi cultivation time (Fig. 1c); the slower the fruiting body harvest was, the higher was the respiration ratio and the larger was the mass loss (Wei et al., 2020). The effects of a single hardwood or wheat straw addition showed no significant difference ($p > 0.05$) in shiitake-based and reishi-based SMS recovery (Fig. 3). The mass of the substrate, regardless of white-rot fungi species or substrate type, decreased slightly during mycelium colonisation and more rapidly within the following two weeks (in-between). The middle and latter stages of fungi cultivation (from in-between to SMS) consumed most substrates, corresponding to >70 % of the total losses.

On the basis of the contents of each component and substrate mass recovery after edible and medicinal fungi cultivation, the relative mass reduction/degradation of lignocellulose was calculated (Fig. 4). Shiitake cultivation resulted in 57.1 % lignin degradation, on average (Fig. 4a), which was 2.7 and 1.5 times higher than the values achieved in oyster (21.0 %) and reishi (38.4 %), respectively. Lignin degradation was positively ($p < 0.05$) correlated with wheat straw addition for the three fungi species and showed the maximal value (61.4 %, 23.2 % and 42 %, respectively) with 10 % or 20 % wheat straw addition.

High xylan and glucan degradation (70.1 % and 58.5 %, on average) was achieved after shiitake cultivation (Fig. 4b and c), followed by reishi, which resulted in 61.6 % xylan degradation and 52.5 % glucan degradation. In contrast to lignin, the difference in carbohydrate degradation between shiitake and reishi was rather small. Wheat straw addition had a quadratic correlation ($p < 0.05$) with carbohydrate degradation. A higher value was found with 10 % wheat straw addition for both shiitake and reishi. Oyster cultivation resulted in as low as

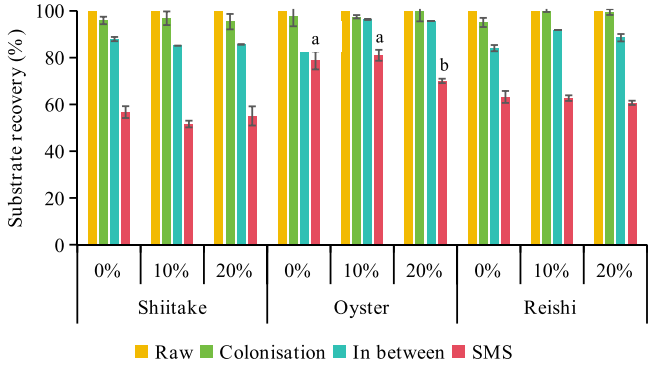

Fig. 3. Mass changes of substrates over different cultivation stages of shiitake, oyster and reishi (mean ± SE). Different letters (a, b) indicate significant differences between treatments ($p < 0.05$).

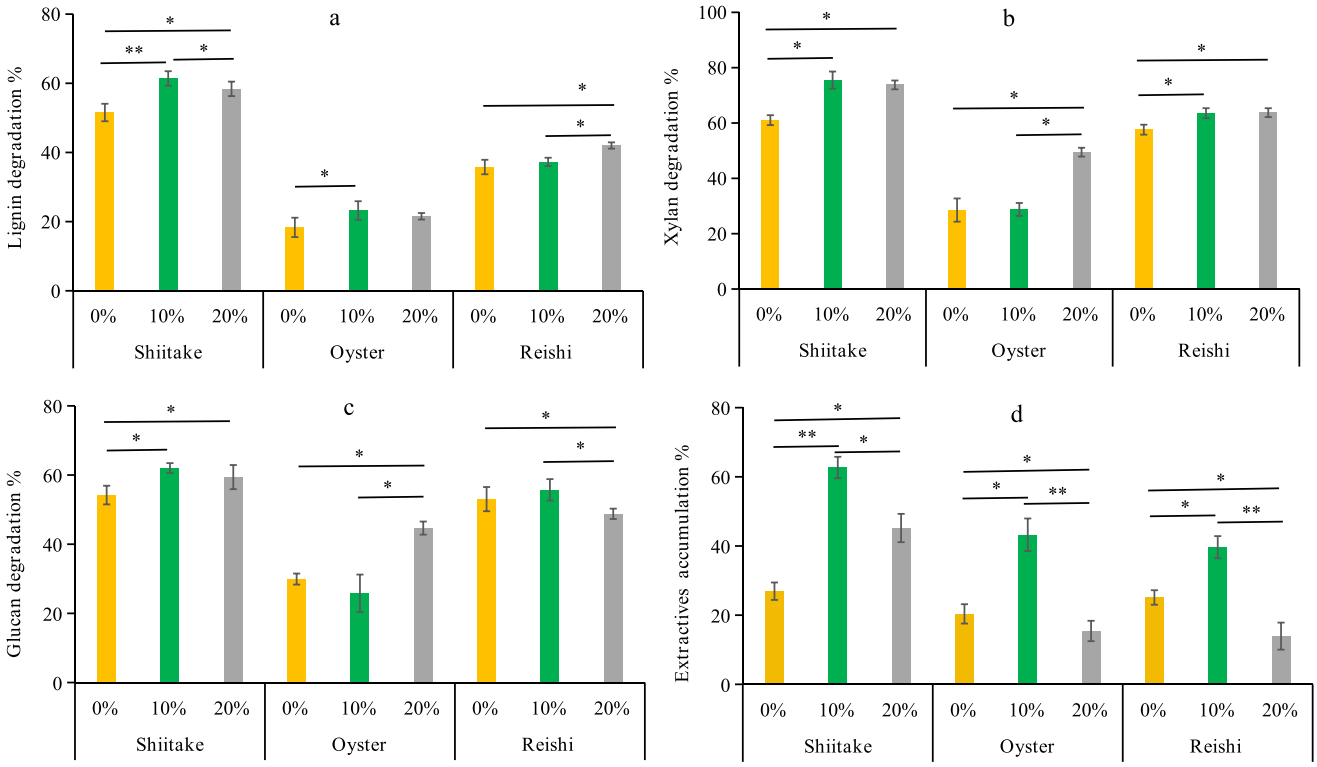

Fig. 4. Mass degradation of lignin (a), xylan (b) and glucan (c), and extractive accumulation (d) in SMSs with respect to the raw substrates (mean ± SE). * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between treatments.

35.6 % and 33.5 % (on average) mass reductions on xylan and glucan, respectively (Fig. 4b and c). In addition, significantly lower ($p < 0.05$) xylan and glucan degradation (28.5 %–28.8 % and 25.8 %–29.9 %, respectively) was observed at 0 % and 10 % wheat straw addition, in contrast to 49.5 % and 44.7 %, respectively, at 20 % wheat straw addition.

Lignin and xylan are considered the main barriers limiting enzymatic hydrolysis for cellulosic ethanol production (Jönsson and Martín, 2016). Remarkably, the mass degradation of lignin and xylan found in this shiitake cultivation was generally comparable to or even higher than the previous studies on birch, alder and aspen treatment with shiitake (Chen et al., 2022a, 2022b; Xiong et al., 2019). However, glucan had a higher rate of degradation than lignin in this shiitake study. The high losses were in contrast to previous studies showing only 20 %–50 % glucan degradation (Chen et al., 2022a, 2022b; Xiong et al., 2019). This might be attributed to the differences in shiitake strains between that used previously and this study (M3790 vs. strain Shenxiang 16). In addition, the shiitake fruiting bodies were harvested ranging from 154 to 186 days in this study (Fig. 1b), and a much shorter time period of 66–130 days was achieved previously (Chen et al., 2022a, 2022b; Xiong et al., 2019). The longer cultivation time, especially the fructification stage, might result in the consumption of carbohydrates (van Kuijk et al., 2015). Reishi had a much higher selective degradation ability on xylan than lignin and glucan. Xylan mass degradation in this study was comparable to shiitake for the same running time (ca. 110 days; (Lin et al., 2015)). Oyster had limited lignin and xylan degradation but with modest glucan losses. The pretreatment potential of oyster on lignocellulose was not obviously different from other white-rot fungi, such as *Pleurotus pulmonarius*, *Echinodontium taxodii* and *Pseudolagarobasidium acaciicola* (Chen et al., 2020; Rudakiya and Gupte, 2017; Yu et al., 2009).

3.2.3. Relative change in the syringyl-to-guaiacyl ratio in lignin

The raw substrates had syringyl-to-guaiacyl (S:G) ratios of 0.65–0.92 (Table 2), which decreased with wheat straw addition. Compared with the lignin of hardwood, which consists of significant fractions of both guaiacyl and syringyl units, the lignin of crop straw consists mostly of guaiacyl units (Chen et al., 2022b; Wang et al., 2018).

Table 2
S:G ratio of lignin in raw substrates and SMSs.

Substrates	S:G ratio of lignin		
	0 %	10 %	20 %
Raw	0.92 ± 0.01	0.85 ± 0.02	0.65 ± 0.02
Shiitake-SMS	0.50 ± 0.04	0.47 ± 0.04	0.47 ± 0.03
Oyster-SMS	0.81 ± 0.00	0.81 ± 0.00	0.76 ± 0.02
Reishi-SMS	0.77 ± 0.01	0.65 ± 0.06	0.61 ± 0.07

After shiitake cultivation, the S:G ratio decreased to 0.47–0.50 in the SMSs, which might be attributed to a higher enzymatic reactivity of S-lignin in the substrates (Table 2). This finding agrees with our previous study showing a 41.5 % reduction in the S:G ratio of hardwoods after shiitake cultivation (Chen et al., 2022b). Manganese peroxidase (MnP) and laccase are the major lignin-degrading enzymes secreted by white-rot fungi (Janusz et al., 2013), but they have different preferences regarding the degradation of lignin units. MnP is capable of oxidising non-phenolic compounds and minor phenolic moieties of lignin, and laccase is a copper-containing oxidase that oxidises numerous phenolic compounds (Janusz et al., 2013; Wan and Li, 2012). S unit-rich lignin often has a low content of free phenolic groups due to their involvement in the formation of methoxy groups (Camarero et al., 1999; Shirkavand et al., 2016). Plausibly, the high reactivity of S-lignin of shiitake can be attributed to more MnP activities compared with laccase activity involved in lignin degradation. However, compared with the substrate composed of a single hardwood, 10 % wheat straw addition resulted in a slightly lower reduction in the S:G ratio but higher lignin mass degradation (Table 2, Fig. 4a). It was hypothesised that laccase activity would increase and be involved in lignin degradation within a certain amount

of wheat straw addition. A considerably lower reduction of S:G ratio (28 %) was observed when adding 20 % wheat straw, in which further inhibition of MnP activity did not favour S-lignin degradation (Fig. 4a), delayed mycelium growth and was unable to complete fruiting body harvest (Fig. 1). However, the explanation of that phenomenon is beyond the scope of the current investigation, and a deeper understanding remains to be studied.

Reishi cultivation decreased the S:G ratio to 0.61–0.77 in the SMSs, and high wheat straw addition was associated with less change of S:G ratio (Table 2), which revealed a similar pattern with shiitake. Differently, reishi achieved a faster mycelium growth with the wheat straw addition, although the fruiting body yields were negatively correlated (Fig. 1), which indicated that reishi was less sensitive to the inhibition of MnP activity.

Except for the single hardwood substrate, which revealed a slight decrease in the S:G ratio, the S:G ratio of the substrates with wheat straw addition remained comparable or even increased after oyster cultivation (Table 2). The effect of oyster on the substrate S:G ratio was in contrast with that of shiitake and reishi. The high reactivity of G-lignin in this study suggests that for oyster mushroom, both MnP and laccase are involved in lignin degradation. The diverse functions of lignin-degrading enzymes might explain why oyster is an aggressive coloniser of a wide array of substrates (Chen et al., 2020; Fernandes et al., 2015) and why it achieved considerable production in wheat straw substrates in this study (Fig. 1).

3.3. Enzymatic hydrolysis of the SMSs

To assess how fungal growth affected the susceptibility of the substrates to cellulolytic enzymes, parallel enzymatic hydrolysis assays were performed for the raw substrates and the SMSs. As indicated in Fig. 5a, the enzymatic digestibility of glucan was rather low for the raw substrates (0.2 %–0.3 %).

Shiitake cultivation resulted in the considerable enhancement of the susceptibility of the substrate to enzymatic saccharification (Fig. 5a). The highest glucan digestibility of the studied SMS reached up to 84.4 % when 10 % wheat straw was added, followed by 20 % and 0 % wheat straw addition (82.6 % and 47 %, respectively). The results confirm previous reports on shiitake cultivation as a pretreatment method for enhancing the enzymatic convertibility of birch cellulose (Chen et al., 2022c; Xiong et al., 2019). The glucan digestibility of the reishi-based SMS ranged from 15.2 % to 33.5 % (Fig. 5a). Although hydrolytic conversion was rather modest, it was greatly higher than the value achieved in the hydrolysis of the raw substrates. By contrast, oyster cultivation had poor performance on lignocellulose pretreatment, and the glucan digestibility of the SMS was as low as 0.3 %–1.5 % (Fig. 5a).

The results of the present study showed that the glucan digestibility of SMS was dependent on the white-rot fungi species and substrate types. On the basis of a 12 × 5 data matrix, including the 12 analytical enzymatic hydrolysis experimental runs and 5 characteristic substrate variables, PCA was performed (Fig. 5b) to identify multi-variable relations. The biplot, composed of the first two PCA components and explaining 93.1 % of the total variation, showed that the observations were visually clustered into three groups that were separated from right to left by the raw substrates and oyster-based, reishi-based and shiitake-based SMSs. The negative effects of lignin and xylan on enzymatic digestibility are anticipated (Jönsson and Martín, 2016; Shirkavand et al., 2016), in line with the highest enzymatic digestibility of the shiitake-based SMS with 10 % wheat straw addition, which had relatively lower lignin and xylan contents (Fig. 2). In addition, the S:G ratio showed a negative effect on enzymatic saccharification (Fig. 5b). This is in contrast to Kraft pretreatment, which showed that the increase in the S:G ratio of lignin increased enzyme adsorption and resulted in higher enzymatic hydrolysis efficiency (Santos et al., 2012).

3.4. Fermentation of hydrolysates in ethanol

The results of the present study showed that shiitake and reishi cultivation could be an effective biological pretreatment in facilitating the enzymatic saccharification of cellulose. In addition, by regulating the addition of wheat straw to the raw substrate, it is possible to regulate the lignocellulose degradation and saccharification of SMSs. The preparative enzymatic saccharification of shiitake-based and reishi-based SMSs with 10 % wheat straw addition (Table 3) and the fermentation of the resulting hydrolysates were performed (Fig. 6).

During the first 2 h of fermentation, approximately 46.6 % of the initial glucose content in the hydrolysate of reishi-based SMS was consumed by *S. cerevisiae*, corresponding to a volumetric consumption rate of 1.64 g/L h (Fig. 6a), and glucose was depleted soon at 4 h. In the case of shiitake-based SMS, glucose was consumed slowly during the first 2 h (1.49 g/L h) and then more rapidly (2.73 g/L h) between 2 and 6 h, which was depleted at 8 h.

The hydrolysate of reishi-based SMS resulted in ethanol volumetric productivities of 0.61 and 0.89 g/L h during the first 2 h and second 2 h, respectively (Fig. 6a). At 4 h of fermentation, the ethanol concentration in the hydrolysate reached its peak (3 g/L), corresponding to a yield of 42.5 g/100 g glucose (Fig. 6b), after which it decreased because of evaporation. For the hydrolysate of shiitake-based SMS, 7.4 g/L ethanol (Fig. 6a), corresponding to a maximal yield of 39.8 g/100 g glucose (Fig. 6b), was achieved at 8 h. It was observed that glucose consumption and ethanol formation were slightly slower during 6–8 h compared with those during 2–6 h, suggesting that fermentation was likely completed before 8 h (Fig. 6a). A relatively higher ethanol yield for the shiitake-based SMS hydrolysate might be expected. However, compared with the volumetric productivity of ethanol in the fermentation of the reishi-based SMS hydrolysate, the values for the fermentation of the shiitake-based SMS hydrolysate were notably lower, at 0.5 and 0.88 g/L h during the first 2 h and second 2 h, respectively (Fig. 6a). For the hydrolysate of shiitake-based and reishi-based SMSs, the ethanol yield corresponded to 78.0 % and 83.2 %, respectively, of the theoretical maximum yield (51.1 g/100 g glucose) (Krishnan et al., 1999), and the variations in fermentability suggest that the fungal pretreatment-derived by-products might cause inhibitory effects on the yeast.

In comparison to structural components such as lignin, hemicellulose and cellulose, extractives usually represent a minor fraction comprising <10 % in raw hardwood (Chen et al., 2022a, 2022b). However, the mass of extractives increased by 13.9 %–62.7 % in SMSs compared with that in the raw substrates (Fig. 4d). The mushroom species and substrate types had significant effects on extractive accumulation, and shiitake cultivation with 10 % wheat straw addition resulted in the largest increase ($p < 0.05$). The highest mass accumulation of extractives was generally associated with the largest degradation of the lignocellulose components. Although the compounds of extractives remain unclear, it is expected that they are composed mostly of the by-products resulting from lignocellulose degradation by the fungus.

The formation and inhibitory effects of lignocellulose degradation–derived by-products by thermochemical pretreatment on yeast fermentation have been widely reported (Jönsson and Martín, 2016; Martín et al., 2018; Stagge et al., 2015). This study performed the analysis of the common thermochemical pretreatment by-products in the SMS hydrolysates (Table 3). Levulinic acid and formic acid were detected with total concentrations of 0.58–1.02 g/L in the hydrolysates, and the formation of acetic acid was found to be negligible (~3 mg/L) during fungi cultivation. The hydrolysates also contained phenolic compounds with concentration of 19–26 mg/L. In addition, higher values of these compounds were detected in the hydrolysate of shiitake-based SMS than that of reishi-based SMS, which was the consequence of higher extractive accumulation (Fig. 4d). The finding indicated that the degree of lignocellulose degradation was positively correlated with by-product accumulation.

However, the fraction of the detected by-products in the SMS

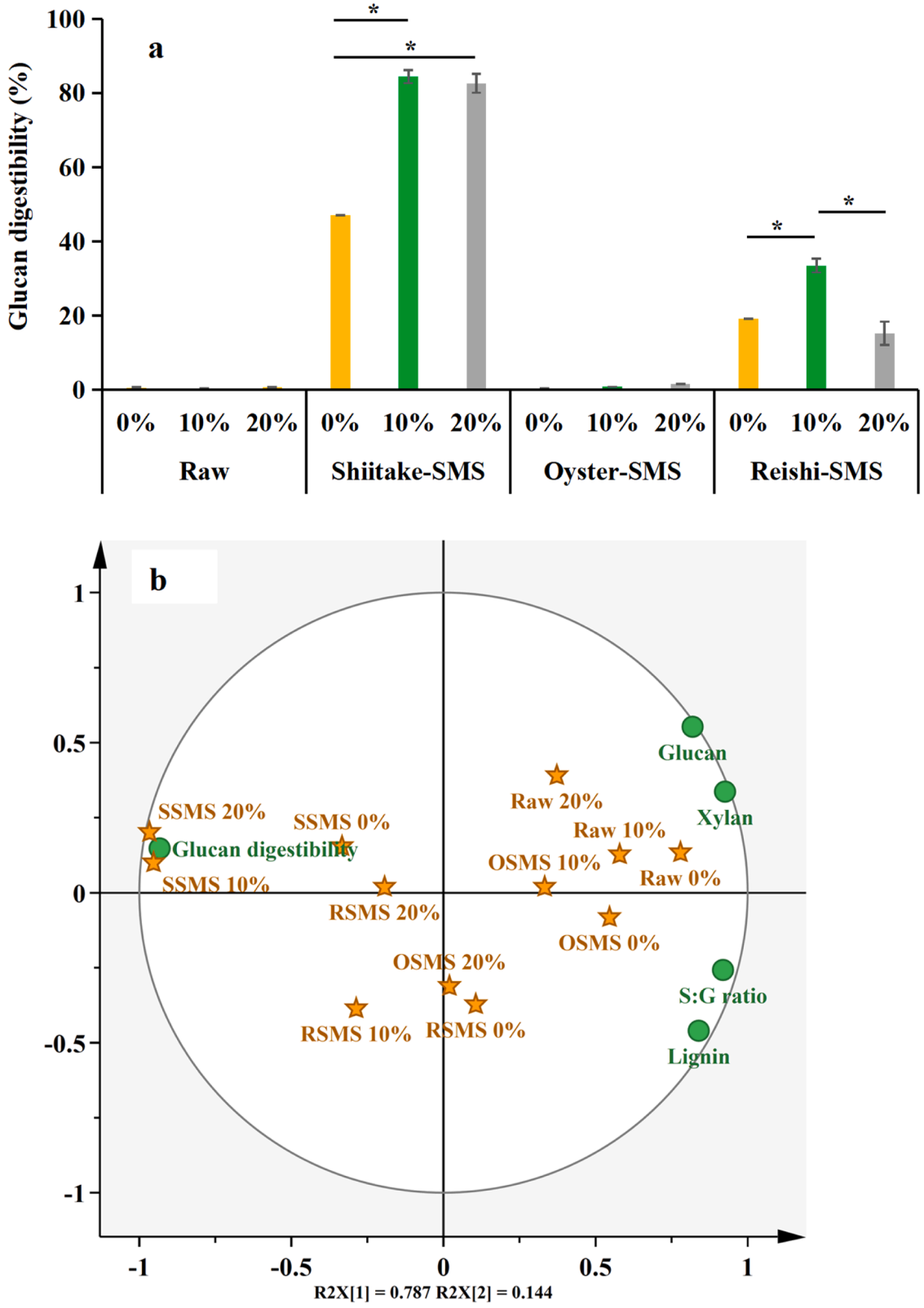

Fig. 5. Enzymatic digestibility (a) of glucan contained in the raw substrates and SMSs (mean ± SE). * indicates significant differences ($p < 0.05$) between treatments. PCA biplot (b) showing the major chemical components of substrates and glucan digestibility. Each star represents an observation. Raw: raw substrate; SSMS: shiitake-based SMS; OSMS: oyster-based SMS; RSMS: reishi-based SMS.

Table 3
Chemical composition of the hydrolysates of shiitake-based and reishi-based SMSs (10 % wheat straw addition). Data refer to mean values ± standard error (SE).

Parameters	Units	Hydrolysates of	
		Shiitake-based SMS	Reishi-based SMS
Glucose	g/L	20.1 ± 0.01	7.6 ± 0.04
Levulinic acid	g/L	0.71 ± 0.2	0.41 ± 0.3
Formic acid	g/L	0.31 ± 0.3	0.17 ± 0.1
Acetic acid	mg/L	3.1 ± 0	3 ± 0
Phenolic compounds	mg/L	26 ± 0.5	19.0 ± 0.8

hydrolysates was below the inhibiting threshold and lower than that in the hydrolysates from thermochemical-pretreated slurries (Du et al., 2020; Ilanidis et al., 2021; Ko et al., 2016; Larsson et al., 1999; Martín et al., 2018), which might have a limited inhibitory effect on ethanolic fermentation. Thus, these might be a significant difference in substrate chemistry following the fungal pretreatment of lignocellulose compared with thermochemical methods. A basic understanding of the characteristics and origin of fungal pretreatment-derived by-products must be gained before large-scale industrial applications can be set.

3.5. *Implications of using edible and medicinal white-rot fungi for biorefinery*

Fig. 7 summarises the mass balance of converting the raw substrate (with 10 % wheat straw addition) (1000 g) to fresh shiitake and reishi fruiting bodies and SMS ethanol. Approximately 51.6 % of the raw substrate remained as SMS after 186 days of shiitake cultivation. After enzymatic saccharification, the obtained glucose, approximately 105.1 g of 1000 g raw substrate, was then fermented to ethanol, yielding 41.8 g. Compared with shiitake, reishi cultivation resulted in the lower degradation of lignin (61.4 % vs. 37.3 %) and xylan (75.5 % vs. 63.6 %), but the glucan losses did not differ (62.0 % vs. 55.7 %) (Fig. 4). The theoretical yields of ethanol converted from the reishi-based SMS were estimated to be 21.1 g per 1000 g DM of raw substrate. The values were

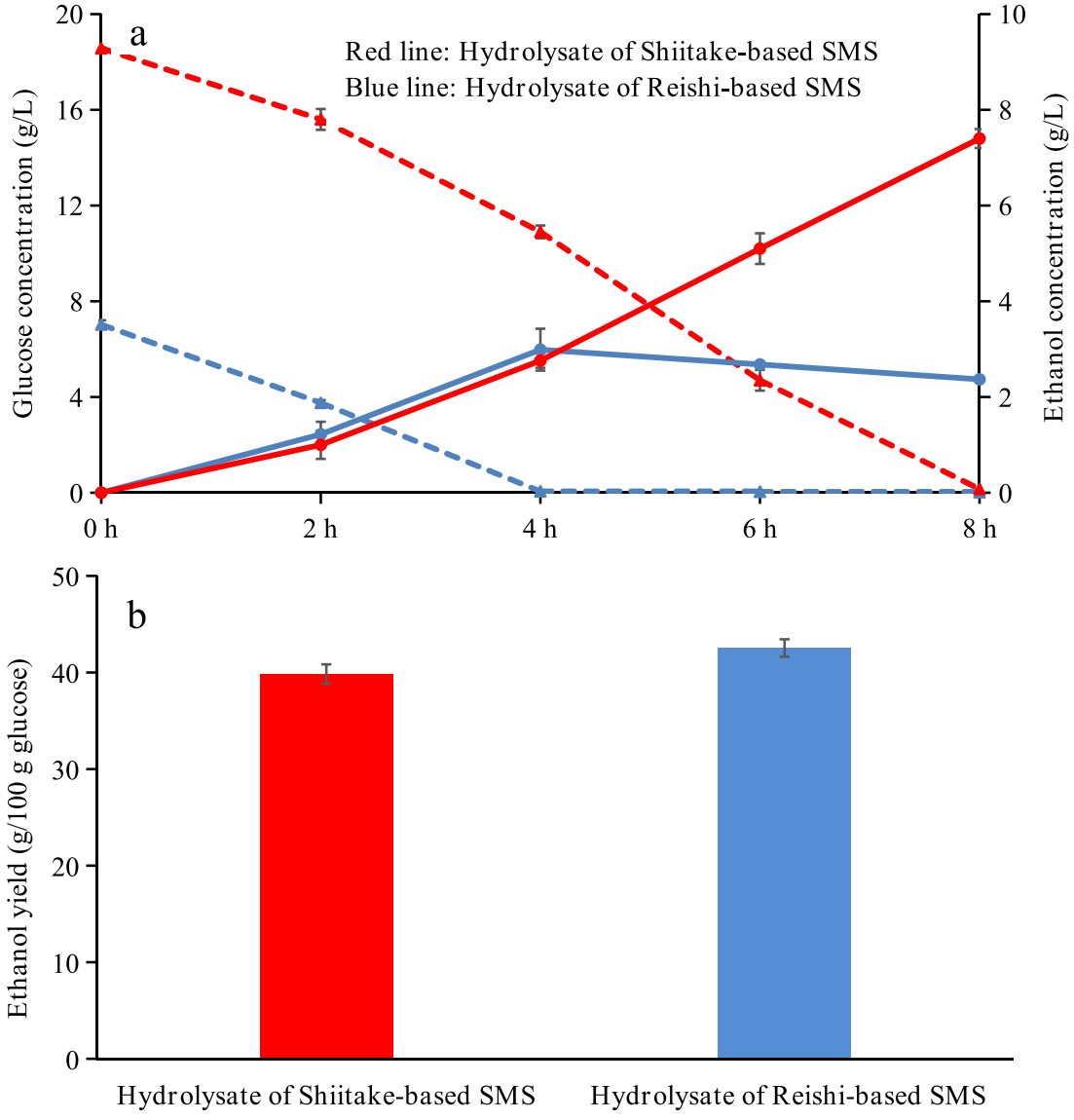

Fig. 6. Glucose consumption (dotted line) and ethanol production (solid line) (a) during *S. cerevisiae* fermentation of hydrolysates of shiitake-based SMS (red) and reishi-based SMS (blue) (10 % wheat straw addition). Ethanol yield (b) of SMS hydrolysates. Data refer to mean values ± standard error (SE).

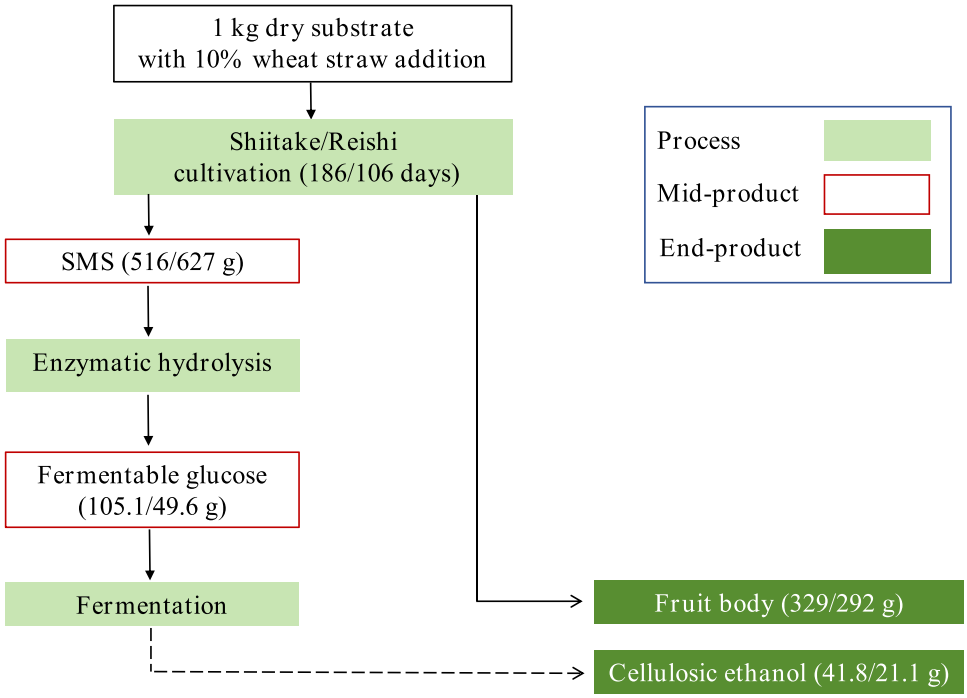

Fig. 7. Schematic of the concept and mass balance for the combined production of shiitake and reishi mushrooms and cellulosic ethanol from a substrate with 10 % wheat straw addition.

almost half those of the shiitake-based SMS. However, the use of reishi may have advantages over shiitake because of the shorter cultivation cycle (186 vs. 106 days, Fig. 1b). The edible and medicinal mushroom industry is developing fast (Royse et al., 2017), and SMS has traditionally been discarded as waste or combusted directly. This reveals the high potential of biological pretreatment using shiitake and reishi as a biorefinery approach, producing cellulosic ethanol and edible and medicinal mushrooms with high value as food and sources of nutraceuticals and pharmaceuticals.

4. Conclusions

Shiitake cultivation as pretreatment, followed by reishi, facilitated lignocellulose bioconversion and resulted in good enhancement of SMS saccharification. Oyster cultivation had poor performance in lignocellulose pretreatment. In contrast to the oyster mushroom, shiitake and reishi had high reactivity of S-lignin. The low S:G ratio of wheat straw might be the major reason for the strong recalcitrance of the 20 % addition for shiitake and reishi cultivation. Compared with the substrate composed of a single hardwood, 10 % wheat straw addition resulted in a generally comparable fruiting body yield and higher lignocellulose degradation and saccharification of SMS. The variations in the fermentability of the resulting hydrolysates suggest that the fungal pretreatment-derived by-products might cause inhibitory effects on yeast.

CRediT authorship contribution statement

Yue Xu: Investigation. **Shaojun Xiong:** Writing – original draft, Investigation, Funding acquisition. **Feng Chen:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Yi Yin:** Writing – original draft, Investigation. **Binbin Chen:** Writing – original draft. **Shuai Xu:** Investigation. **Jinchen Zuo:** Investigation.

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.

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Data Availability

Data will be made available on request.

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