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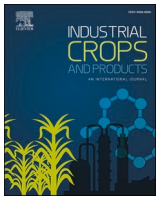

Fungal pretreatment of hardwood for cellulosic ethanol production: Formation of by-products and the potential effects on downstream bioconversion processes

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ABSTRACT

Shiitake mushroom cultivation could effectively reduce the recalcitrance of hardwood, facilitating cellulosic ethanol production. However, the shiitake pretreated spent mushroom substrates (SMS) have exhibited significant variations in enzymatic saccharification and fermentation efficiencies in previous studies. Parallel with shiitake cultivation, significant increases in extractives mass were observed in the SMS. This highlights the need to examine the formation of pretreatment by-products and their potential impact on downstream bioconversion processes. In this study, shiitake cultivation resulted in 42.6–47.6 % degradation of lignocellulosic components, with continuous generation of various small molecules. Shiitake demonstrated extensive and selective utilization of these degradation products during fructification. Consequently, non-utilized molecules, including (L)-dehydroascorbic acid, triglochinic acid, and 5-hydroxy-2-methylchromone, accumulated in SMS extractives as pretreatment by-products. The SMS cellulose showed 58.8 % digestibility upon enzymatic saccharification. The resulting hydrolysate was fermented to ethanol by *Saccharomyces cerevisiae* rendering 83.4 % of the theoretical yield. Although water extraction effectively minimized the by-products accumulation in SMS and hydrolysate, no improvement of enzymatic saccharification and fermentation efficiencies were observed. In contrast to thermochemical pretreatment methods, the by-products derived from shiitake pretreatment had limited inhibitory effects on downstream bioconversion processes. This study provides valuable indications for further optimization of shiitake pretreatment towards industrial implementation.

1. Introduction

Cellulosic ethanol, produced by enzymatic saccharification and fermentation of lignocellulosic biomass, has received considerable attention as a renewable alternative to fossil fuels. Pretreatment is a prerequisite step for removing lignin and hemicelluloses enabling cellulose amenable to enzymatic saccharification (Kumari and Singh, 2018; Mankar et al., 2021). Fungal pretreatment using shiitake (*Lentinula edodes*) mushroom has shown to be a prominent strategy. After growing shiitake on hardwoods, considerable degradation of lignin and hemicelluloses (up to 67.6 % and 61.3 %, respectively) have been observed in the spent mushroom substrate (SMS) (Chen et al., 2022b; Yin et al., 2025). Enzymatic saccharification assays revealed that the digestibility of cellulose contained in the SMS reached up to 48.0–92.8 % (Chen et al., 2022a, 2022c; Lin et al., 2015; Xiong et al., 2019; Yin et al., 2025). Subsequently, the SMS hydrolysates were fermented to ethanol yielding 38.5–44.4 g/100 g glucose (Chen et al., 2022c; Yin et al., 2025).

The edible mushroom industry is developing fast (Royse et al., 2017), and SMS has traditionally been discarded as waste or combusted directly. As research progresses, an increasing number of studies explore the unique characteristics of SMS from various mushrooms, while also summarizing methods for its utilization, including biofertilizer, animal feed, renewable energy, and pollution bioremediation (Leong et al., 2022; Ma et al., 2025), as well as for the recovery of enzymes and bioactive compounds (Martín et al., 2023). Shiitake, which is the most cultivated mushroom in the world, generates up to 12.5 million tons SMS per year (Wei et al., 2020). The potential for producing cellulosic ethanol from shiitake industry residues is huge.

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However, a significant variability in enzymatic saccharification (48.0–92.8 %) and fermentation efficiencies (38.5–44.4 g/100 g glucose) was observed in above-mentioned reports. Such variability would be the major drawback of shiitake pretreatment for potential biorefinery industrial application. Although significant efforts have been devoted to facilitating the degradation of lignin and hemicelluloses by optimization of shiitake cultivation (Chen et al., 2022a, 2022b; Xiong et al., 2019; Yin et al., 2025), the side effects and their subsequent impacts on downstream bioconversion processes have been poorly investigated. Understandably, shiitake degrade lignocellulose into available small molecules through its enzymatic system, such as oxidoreductases and carbohydrate-active enzymes (CAZymes), to support the growth of mycelium and fruit bodies (Floudas et al., 2012). The small molecules might accumulate in the SMS as pretreatment by-products, which could potentially inhibit enzymatic saccharification and fermentation processes, and cause their variabilities. This hypothesis is supported by the observed accumulation of extractives in the shiitake growing substrates (Chen et al., 2022a, 2022b; Xiong et al., 2019; Yin et al., 2025). In comparison to structural components such as lignin, hemicelluloses and cellulose, the extractives usually represent a minor fraction, comprising $<$ 10 % in the raw hardwood. The mass of extractives can increase dramatically after the shiitake cultivation, and it reached values that are 1.6–2.0 higher in the SMS than in the raw substrates. As much as 30 % of the SMS was constituted by extractives, with water-soluble components representing the major share ($>$ 95.5 %). Although the composition of the extractive compounds remains unclear, it is expected that they are primarily composed of the by-products formed from lignocellulose degradation.

In contrast to by-products from fungal pretreatment, the formation of by-products from thermochemical pretreatment methods have been widely investigated, and different strategies to alleviate the inhibition problems have been proposed (Jönsson and Martín, 2016). For example, under the acidic conditions, the split of lignin macromolecules and hydrolysis of carbohydrates result in the formation of a high number of phenylic compounds, furfural, 5-hydroxymethylfurfural (HMF), and aliphatic carboxylic acids. Formaldehyde and benzoquinones can further form through the oxidation of some phenolic compounds (Martín et al., 2018; Stagge et al., 2015; Wang et al., 2018). Acetic acid, resulting from the hydrolysis of acetyl groups of hemicelluloses, is a typical by-product of alkaline treatments. Hydroxy acids, dicarboxylic acids, and phenolic compounds are also found in high concentrations in the hydrolysates (Jönsson and Martín, 2016; Kim et al., 2016). The concentrations of these by-products in hydrolysates can be sufficiently high to cause severe inhibition of enzymes and fermentative microorganisms (Jönsson and Martín, 2016; Kim et al., 2016).

Our previous study has preliminarily investigated the accumulation of dominant thermochemical by-products in shiitake pretreated hardwoods (Chen et al., 2022b). In the study, the fraction of soluble phenolic compounds in SMS ranged from 1.8 % to 2.4 %, corresponding to a 1.4–7.2 times increase, respectively, compared to the content in the raw substrate (Chen et al., 2022b). The same study revealed that the furan aldehydes furfural and HMF were not detected in the SMS from shiitake cultivation. Furthermore, the formation of levulinic acid and formic acid was found to be negligible, while acetic acid was detected in low amounts ($<$ 0.1 %) (Chen et al., 2022b). Fungal pretreatment is performed by enzymes, which indicates a significant difference in substrate chemistry and by-products following fungal pretreatment of lignocellulose compared to thermochemical methods. A full understanding of the formation of by-products and their potential effects on downstream bioconversion processes is pivotal to provide indications for further optimization of shiitake pretreatment towards industrial implementation. That knowledge is still lacking.

In this investigation, a hardwood mixture-based substrate was pretreated using shiitake mushroom, and the growing substrates were collected over the cultivation period for the analysis of carbohydrates and lignin. Non-targeted metabolomic analyses were then conducted on these growing substrates to characterize the small organic molecules substances and further identify the key shiitake pretreatment by-products, as well as on the SMS and water-extractive-free SMS (WEF-SMS) to investigate the accumulation of by-products in the extractives. Additionally, enzymatic saccharification of SMS and WEF-SMS was performed, and the fermentability of the resulting hydrolysates with *Saccharomyces cerevisiae* was evaluated in order to understand whether, and to what extent, the by-products affect the downstream bioconversion processes.

2. Materials and methods

2.1. Shiitake cultivation/pretreatment

2.1.1. Preparation of substrates

The hardwood mixtures with particle size of 5–10 mm were sourced from a mushroom grower. Wheat bran ($\leq$ 2 mm) was obtained to serve as a basic nutrient source. The substrates preparation followed usual industrial practices. The substrates were prepared by mixing 80 % hardwood chips and 20 % wheat bran (based on dry mass, DM). Water was then added to achieve a moisture content of approximately 50 %. The pH of the substrates was adjusted to approximately 6.5 by adding 1 % lime of substrate DM. After thorough blending of all ingredients, 250 g of moistened substrate was packed into polypropylene bags with gas-permeable filter suitable for mushroom production. The bags containing the substrates were pasteurized in an autoclave using pressurized steam (121℃ and 2 bar for 2 h) and left overnight in a laminar-flow cabinet to cool to room temperature.

2.1.2. Shiitake mushroom cultivation and sampling

The shiitake mushroom strain 212 used in the experiment was obtained from a local mushroom company as an agar culture. Spawn were produced by propagating the strain on sterile rye grain at 24℃ for 20 days. The inoculation process was performed in a laminar-flow cabinet. Each substrate bag was inoculated with 6.7 g of the inoculum (2.7 % of wet substrate mass), and the bags were placed in an incubation room in the darkness at a temperature of approximately 24℃. Once the mycelia successfully ripened and a brown crust appeared on the substrate cylinder surface, 10 holes were punctured through the plastic bag around the lateral area of each synthetic log of the substrate, allowing for fruiting. The fructification phase was induced using light ($<$ 300 lx), an air temperature of 18/24℃ (12 h/12 h), and 80 % relative humidity until the harvest was completed (one harvest only).

The substrate samples were collected as follows: (1) Raw substrates were collected immediately after blending but before the sterilization; (2) Intermediate samples were collected on Day 21, 42, and 63 of incubation, with three bags sampled for each time point to ensure statistical robustness; (3) The thirteen replicated bags were used for harvesting and estimating the yield of fruit bodies, after which the SMS was collected. The collected substrates were dried at 45℃ to constant weight, then milled to $\leq$ 0.5 mm, and stored in airtight plastic bags at room temperature.

2.2. Preparation of WEF-raw substrate and WEF-SMS

To obtain the water-extractive-free raw substrate (WEF-raw substrate) and water-extractive-free SMS (WEF-SMS), water extraction was performed on the raw substrate and SMS following the NREL protocol (Sluiter et al., 2005), with some modifications. For each extraction, approximately 5 g (DM) of sample was placed in a cotton cellulose thimble and successively extracted with 100 mL water for 6 h using a Soxhlet apparatus with heating temperature at 150℃. Once the reflux time was complete, the WEF materials were air-dried and stored in airtight plastic bags at room temperature. Approximately 100 g (DM) WEF materials were collected in total for each sample to be used in the following analysis and experiment.

2.3. Enzymatic saccharification

2.3.1. Analytical enzymatic saccharification

The analytical enzymatic saccharification of the raw substrate, SMS, WEF-raw substrate, and WEF-SMS were assayed as previously described (Chen et al., 2022c; Gandla et al., 2018), with some modifications. For each sample, 0.5 g DM was suspended in 5 mL of 50 mM sodium citrate buffer (pH 5.2) in 10-mL tubes. Subsequently, commercial enzyme preparation Cellic CTec3 (initial enzyme activity: 340 CMCase units/mL), a blend of cellulases, β-glucosidases, hemicellulases, and LPMOs (lytic polysaccharide monooxygenases) acquired from Novozymes Investment Co Ltd, Beijing, China, 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. At the end of the 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 treatment was conducted in triplicate, and the mean values and standard error (SE) were reported.

2.3.2. Preparative enzymatic saccharification

Preparative enzymatic saccharification of the SMS and WEF-SMS was performed in order to produce hydrolysates to be used in the fermentation experiment (Chen et al., 2022c). The process is rather similar as the analytical enzymatic saccharification, but the scale is different. Namely, in the preparative enzymatic saccharification, 20 g of the biomass sample was suspended in sodium citrate buffer at 10 % solids content in 500-mL Erlenmeyer flasks. After hydrolysis and centrifugation, the hydrolysate was adjusted to pH 5.5 with NaOH solution and filter-sterilized through 0.22 μm sterile filtration unit under vacuum.

2.4. Fermentation of hydrolysates

2.4.1. Inoculum and media

To prepare the inoculum, freeze-dried yeast (*S. cerevisiae* YSC2, Sigma-Aldrich, Trading Co. Ltd, Shanghai) was suspended in sterile deionized water at 35 °C for 30 min. The yeast concentration in each fermentation flask was 1 g/L. The nutrient solution, corresponding to 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 $MgSO_4{\cdot}7H_2O$, 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-sterilized hydrolysate of SMS or WEF-SMS, 5.6 % (v/v) yeast inoculum, and 2 % (v/v) nutrient solution.

2.4.2. Fermentation

The fermentation of the hydrolysates of SMS and WEF-SMS were assayed as previously described (Chen et al., 2022c; Yin et al., 2025), with some modifications. Filter-sterilized 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 2.5, 5, 7.5, and 10 h, according to previously established methods (Chen et al., 2022c; Yin et al., 2025) and pilot test results. The ethanol yield was calculated as the maximum amount of ethanol formed per 100 g of initial glucose. The cell growth of *S. cerevisiae* was monitored by OD (optical density) measurements (Martín et al., 2018) at 600 nm using a spectrometer (Shimadzu, Kyoto, Japan). The OD was measured after 0, 2.5, 5, 7.5, 10, 12.5 and 15 h. Each experimental treatment was conducted in quadruplicate, the mean values and standard error (SE) were reported.

2.5. Analytical methods

2.5.1. Metabolite analysis

A non-targeted metabolomics analysis was adopted to assay metabolites in the raw substrate, Day 42 substrate, SMS and WEF-SMS, as well as the filter-sterilized hydrolysates of SMS and WEF-SMS. The substrate samples were ground with steel balls in a tissue grinder, and 50 mg of the powdered sample was suspended in a 600-μL pre-cooled mixture of methanol and 2-amino-3-(2-chloro-phenyl)-propionic acid (4 ppm), vortexed for 30 s, and incubated for 15 min under ultrasound at room temperature. After that, the extraction mixture was separated by centrifugation (12,000 rpm at 4 °C for 10 min), filtered (0.22 μm), and subjected to liquid chromatography mass spectrometry (LC-MS) analysis. The liquid chromatography was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA), equipped with an ACQUITY UPLC T3 column (Waters, Milford, MA, USA). Mass spectrometric detection of metabolites was performed on Orbitrap Exploris 120 (Thermo Fisher Scientific, USA) with ESI ion source. Metabolites were identified based on the spectral data, with reference to public databases including the Human Metabolome Database (HMDB, http://www.hmdb.ca) and METLIN (https://metlin.scripps.edu). The analysis was performed at Shanghai Sanshu Biotechnology Co., Ltd (Shanghai, China).

2.5.2. Compositional analysis of substrates, hydrolysates, and fermentation samples

Before chemical analysis, the replicated substrates were pooled into one mixed sample. The determination of extractive contents was performed by successive extractions with water and ethanol, according to the NREL protocol (Sluiter et al., 2005). Extractive-free materials were air-dried and used for the analysis of structural carbohydrates and lignin by analytical acid hydrolysis combined with high-performance liquid chromatography (HPLC) (Sluiter et al., 2008).

The mass degradation of major components from the initial mass could then be calculated using the following equation: Relative mass degradation % = $[1 - (M_J * C_J)/(M_0 * C_0)] * 100$, where M and C refer, respectively, to mass of substrate and content of component (extractives, lignin, glucan and xylan) on growing Day J (21, 42, 63, SMS) and Day 0 (raw), respectively.

The concentrations of glucose, xylose, levulinic acid, formic acid, acetic acid, and ethanol in the enzymatic hydrolysates and fermentation samples were determined by HPLC, using an Aminex HPX-87H column and a RI detector. Elution was performed with an isocratic flow of a 5 mM aqueous solution of sulfuric acid at a flow rate of 0.6 mL/min and a column temperature of 55 °C (Chen et al., 2022c). Quantitation was performed using external calibration curves with the following concentration ranges: 0–25 g/L for glucose and xylose, 0–2 g/L for levulinic acid, formic acid, and acetic acid, and 0–10 g/L for ethanol. Total phenolic compounds were quantified colorimetrically by the Folin--Ciocalteu's method with vanillin as calibration standard. Total nitrogen was determined by the Kjeldahl method.

2.6. Data analysis

Principal component analysis (PCA) was performed using SIMCA 14.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden) to provide an overview of the metabolites' profiles. Based on the identification criterion of a P value < 0.05 and a fold change (FC) value > 1.5 or < 0.67 from univariate statistical analysis, differential metabolites between substrates were screened. Subsequently, the top 40 metabolites exhibiting the greatest differential abundance (including both up- and down-regulated metabolites) between substrates were selected based on the values of $\log_2$ (fold change).

3. Results

3.1. Shiitake mushroom production

The substrate was fully colonized by shiitake mycelia on approximately 42 days after inoculation, and fruit bodies were harvested between 75 and 81 days (Table S1). The yields of fresh fruit bodies (one flush only, ~ 90 % moisture content) were comparable for all the collected batches, reaching mean values corresponding to 342 g per substrate bag (Table S1).

3.2. Major changes of substrate lignocellulose

The comparison of the composition of the raw substrate, the intermediate substrates collected during the cultivation (on Days 21–63), and the SMS reveals that shiitake cultivation led to a general reduction in the content of lignin (Klason lignin and acid-soluble lignin), xylan, and glucan in the substrates over time (Table 1). The contents of lignin and xylan in the SMS were on average 20.1 % and 12.6 %, respectively, corresponding to a reduction of 15.5 % and 23.2 %, respectively, compared to initial values. Glucan content in the SMS was 16.3 % lower than in the raw substrate. The increase in extractives over time was considerable, with as much as 22.3 % extractives constituting the SMS by the end of shiitake cultivation.

Based on the substrate mass recovery and the contents of each component (Table 1), the relative mass changes during each cultivation stage were calculated (Fig. 1). The average mass degradation of lignin, xylan, and glucan in the SMS after 75–81 days of shiitake cultivation was 42.6 %, 47.6 %, and 43.1 %, respectively, relative to their initial values. Additionally, degradation trends varied with the cultivation stages. It was observed that, compared to lignin and xylan, which degraded rapidly during the first 63 days (accounting for > 75 % of the total degradation), as much as 40 % of the total degradation of glucan occurred between Day 63 and SMS.

As shown in Fig. 1, the major accumulation of extractives occurred during the first 21 days, after which the changes were smaller. During the middle stage of shiitake cultivation (Day 42–63), the accumulation of extractives in substrates reached a maximum, corresponding to approximately a 51.5 % increase compared to that in the raw substrates. The mass of extractives in the substrates then decreased slightly by the end of shiitake cultivation (Day 63 to SMS).

3.3. Metabolic profile analysis of substrates

3.3.1. Chemical taxonomy of substrate metabolites

A total of 1066 metabolites were detected in the raw substrates, Day 42 substrate, SMS, and WEF-SMS in this study, which were classified into 84 chemical compound groups. The 15 most abundant chemical compound groups, sorted by the numbers of composed metabolite species, are shown in Fig. 2. In six groups, the number of metabolite species reached over 40. The groups included prenol lipids (88–172 species), carboxylic acids and derivatives (44–99 species), organooxygen compounds (70–93 species), flavonoids (50–65 species), fatty acyls (49–64 species), and steroids and steroid derivatives (13–45 species). Shiitake

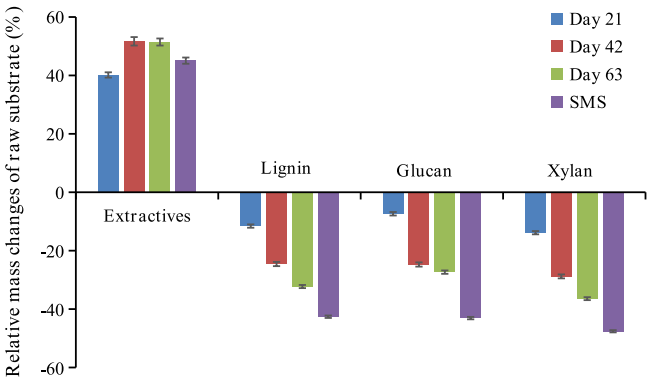

Fig. 1. Relative mass changes of substrate major components on Days 21, 42, and 63 substrates and SMS (Days 75–81) compared to raw substrate. Data refer to mean values ± standard error (SE).

cultivation had a minor impact on the numbers of metabolite species in the substrates. However, water extraction effectively removed certain metabolites from SMS (Fig. 2). For example, metabolite species of prenol lipids, carboxylic acids and derivatives, steroids and steroid derivatives, and glycerophospholipids decreased by more than 46 % in the WEF-SMS.

3.3.2. The differential metabolites between the substrates

The data matrix of 1066 metabolites × 12 substrates was analyzed by PCA to illustrate the trend of metabolomic separation among groups of raw substrate, Day 42 substrate, SMS, and WEF-SMS (Fig. 3). The first principal component (PC1) explained 63 % of the total variation, effectively separating the substrates with and without water extractives. Along the second principal component (PC2, 23.4 %), substrates collected at different cultivation stages were separated from bottom to top. The metabolites of the Day 42 substrate and SMS were visually clustered together, whereas those of the raw substrate formed a distinct cluster of their own.

Based on the identification criterion of a P value < 0.05 and a fold change (FC) value > 1.5 or < 0.67 from univariate statistical analysis, differential metabolites between substrates were screened (Table 2). A total of 943 differential metabolites were observed in the Day 42 substrate versus the raw substrate, with 322 up-regulated and 621 down-regulated. Between the SMS and Day 42 substrate, there were 570 differential metabolites, with 183 up-regulated and 387 down-regulated. For the SMS versus raw substrate comparison, 938 differential metabolites were observed, with 298 up-regulated and 640 down-regulated.

Fig. 4 shows the top 40 differential metabolites between the four studied substrates based on the $\log_2$ (fold change) value. Shiitake mycelium colonization led to significantly higher levels of (L)-dehydroascorbic acid, triglochinic acid, and 5-hydroxy-2-methylchromone, etc., in the Day 42 substrate compared to the raw substrate (Fig. 4a). Compared with the Day 42 substrate, the levels of succinic acid, leucenol, and 6-methxymellein, etc., were significantly up-regulated during the later period of shiitake cultivation (Fig. 4b). Conversely, metabolites including Leu-Val-Ala, N-Fructosyl isoleucylglutamate, and Calystegine

Table 1
Substrate mass recovery and contents of the major components in the raw substrate, substrates collected on Days 21–63, and SMS (Days 75–81). Data refer to mean values ± standard error (SE).

Analysis		Substrates				
		Raw substrate	Day 21	Day 42	Day 63	SMS
Extractives	%	10.4 ± 0	15.9 ± 0.8	18.9 ± 0	20 ± 0.9	22.3 ± 0.4
Lignin	%	23.8 ± 0.6	22.9 ± 0	21.4 ± 0.4	20.4 ± 0.7	20.1 ± 0.3
Glucan	%	32.6 ± 0	32.9 ± 1.0	29.3 ± 0.5	29.9 ± 0.9	27.3 ± 0.2
Xylan	%	16.4 ± 0.5	15.4 ± 0.5	14 ± 0.1	13.2 ± 0	12.6 ± 0
Mass recovery	%	100	91.8 ± 0.6	83.7 ± 0.8	79.1 ± 0.6	68 ± 0.5

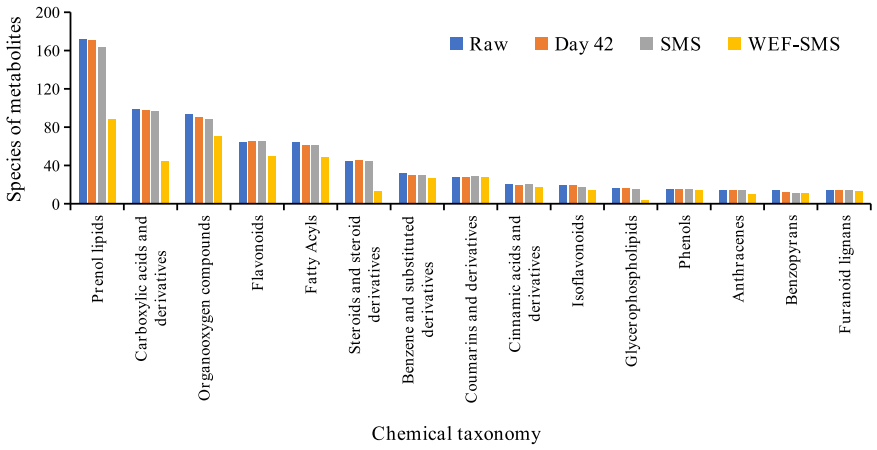

Fig. 2. The 15 most abundant chemical compound groups sorted by the numbers of metabolite species in the raw substrate, Day 42 substrate, SMS, and WEF-SMS.

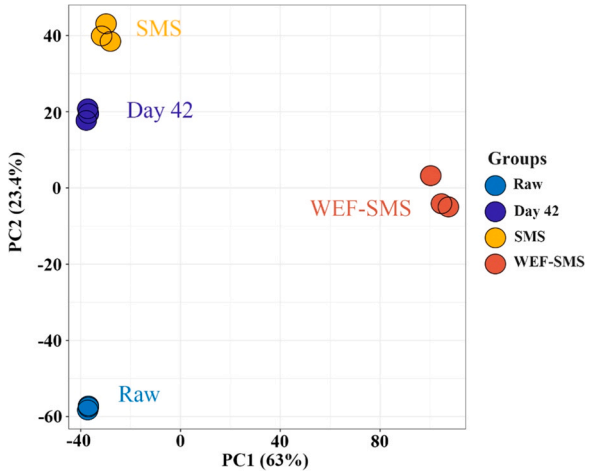

Fig. 3. PCA score plot of the metabolites in the raw substrate, Day 42 substrate, SMS, and WEF-SMS.

Table 2
Differential metabolites between raw substrate, Day 42 substrate, SMS, and WEF-SMS.

Substrates	Total metabolites	Differential metabolites	Up	Down
Day 42 vs. Raw	1062	943	322	621
SMS vs. Day 42	1062	570	183	387
SMS vs. Raw	1062	938	298	640
WEF-SMS vs. SMS	1062	801	288	513

N1, demonstrated marked down-regulation during this period (Fig. 4b). When comparing the differential metabolites over the entire cultivation process (Fig. 4c, Table S2), compared to raw substrates, the SMS contained considerably higher levels of carboxylic acids and derivatives (including Ala-His and triglochinic acid), cinnamic acids and derivatives (including caffeic acid, caftaric acid, and 4-acetoxycinnamic acid), flavonoids (including myricetin-3-O-galactoside and centaureidin), organooxygen compounds (including ribonolactone, delta-gluconolactone, and arbutin), benzene and substituted derivatives (including bergenin), and benzopyrans (including 5-hydroxy-2-methylchromone and 6-methoxymellein), etc.

There were 801 differential metabolites between the WEF-SMS and SMS, with a total of 513 metabolites being down-regulated (Table 2). Compared to SMS (Fig. 4d, Table S2), WEF-SMS exhibited significant decreases in the levels of carboxylic acids and derivatives (including calcium pantothenate, vitamin B15, Ala-His, and triglochinic acid), benzene and substituted derivatives (including bergenin), prenol lipids (including nuezhenidic acid and tirotundin), cinnamic acids and derivatives (including caftaric acid), and coumarins and derivatives (including 3-hydroxycoumarin), etc.

3.4. Enzymatic saccharification of substrates and chemical compositions of hydrolysates

To assess the effects of shiitake pretreatment and by-products on the substrate's susceptibility to cellulolytic enzymes, enzymatic saccharification assays were performed for the raw substrate, WEF-raw substrate, SMS, and WEF-SMS. As indicated in Fig. 5, the enzymatic digestibility of glucan was rather low for the raw substrate (1.9 %). Shiitake pretreatment enhanced the susceptibility of the substrate to enzymatic saccharification, leading to a glucan digestibility of up to 58.8 % in the SMS (Fig. 5). The water extraction had a positive effect for the raw substrate on enhancing the glucan digestibility to 5.3 %. The WEF-SMS demonstrated comparable efficiency to SMS, achieving a glucan digestibility of 58.6 %.

The enzymatic hydrolysates obtained from SMS and WEF-SMS yielded glucose concentrations of 16.5 g/L and 20.4 g/L, respectively (Table 3). The hydrolysate of WEF-SMS had a higher glucose concentration compared to SMS, reflecting an increase in glucan content after water extraction and comparable glucan digestibility (Fig. 5). The hydrolysis resulted in xylose concentrations of 4.8 and 5.6 g/L, respectively, in the hydrolysates of SMS and WEF-SMS.

The dominant thermochemical by-products were investigated in the hydrolysates of SMS and WEF-SMS (Table 3). Levulinic acid and formic acid were hardly detectable in the shiitake substrates, but were found in the hydrolysates at concentrations of 0.51–0.62 g/L and 0.05–0.15 g/L, respectively. Acetic acid was not detected in the hydrolysates. The phenolic compounds were present in concentrations of 0.42–0.77 g/L. Lower concentrations of the above components were found with WEF-SMS hydrolysate. The total nitrogen concentration was as high as 858.4 mg/L in the SMS hydrolysate. The corresponding value in WEF-SMS hydrolysate was substantially lower at 435.4 mg/L, representing only half the concentration observed in SMS hydrolysate.

A total of 560 metabolites were detected in the hydrolysates, with 440 of them showing a significant difference between WEF-SMS and SMS, with 257 being up-regulated and 183 being down-regulated (data not shown). Compared to the hydrolysate of SMS (Fig. 6, Table S3), that of WEF-SMS exhibited significant decreases in the levels of peptide chains, such as Ala-Tyr-Ile-Asp, Ile-Glu-Leu-Lys, Val-Tyr-Thr, and Ile-Ile-Ser, among others. Conversely, significant accumulations of benzene and substituted derivatives (including vanillic acid, gallic acid, and phthalic acid), organooxygen compounds (including delta-

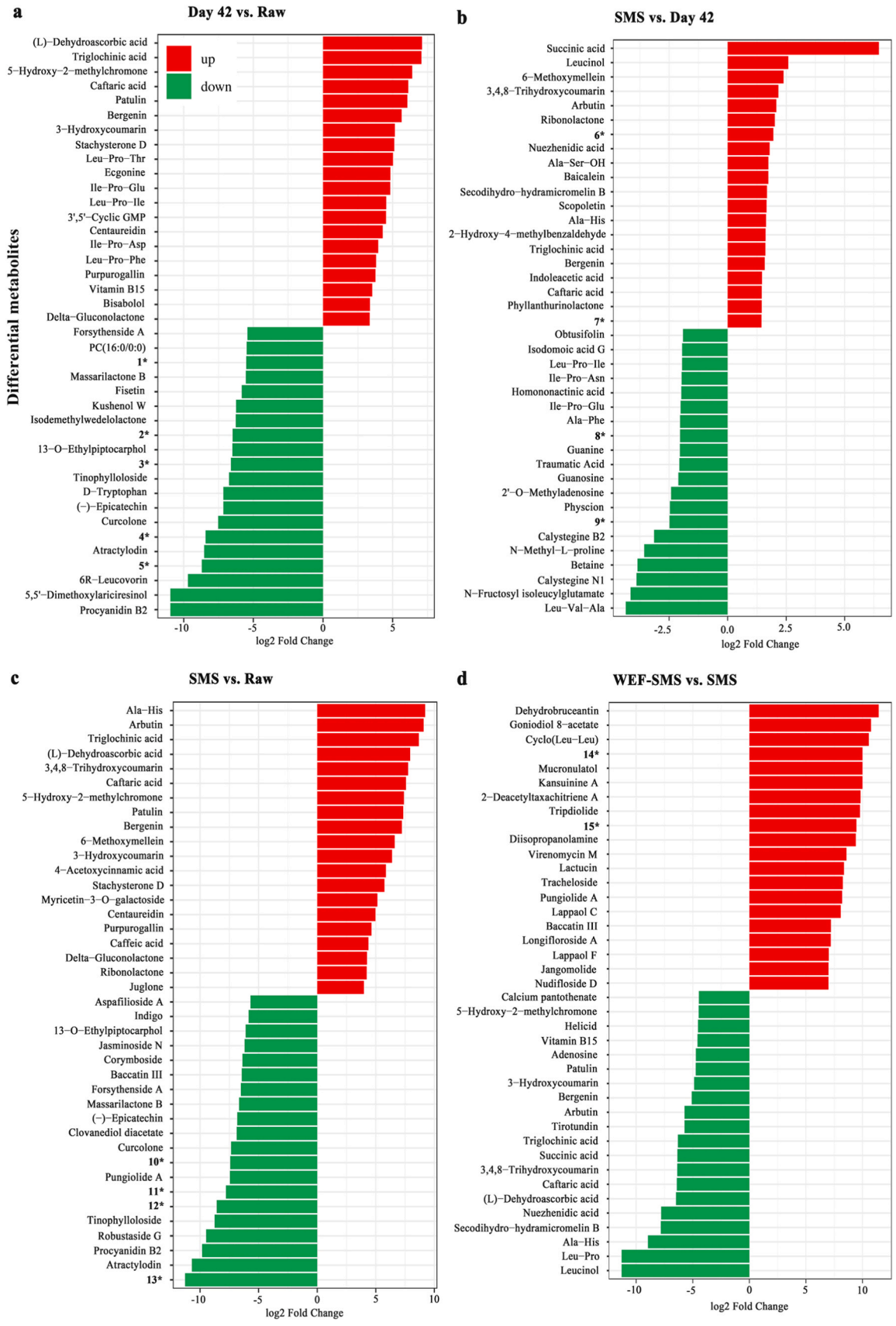

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Fig. 4. Detection and analysis of metabolites in the raw substrate, Day 42 substrate, SMS, and WEF-SMS. **a-d**: the top 40 differential metabolites between substrates based on the log_2 (fold change) value. **1*** 2,2′-(3-methylcyclohexane-1,1-diyl)diacetic acid, **2*** (1R,6R,9R)-6,9,11-trihydroxy-4,7-megastigmadien-3-one-11-O-glucoside, **3*** 8alpha-methacryloyloxy-13-ethoxyvernojalcanolide, **4*** 8-epi-loganic acid-6′-O-alpha-D-glucoside, **5*** (2*E*)-2-[2-(1,2,4a,5-tetramethyl-1,2,3,4,a,7,8,8a-octahydro-1-naphthalenyl)ethyl]-2-butenedioic acid, **6*** 2-(2-(2,5-dioxohexahydro-imidazo[4,5-*d*]imidazole-1(2H)-yl)acetamido)acetic acid, **7*** ethyl 2-(1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)acetate, **8*** 3-(benzoyloxy)-2-hydroxypropyl-D-glucopyranosiduronic acid, **9*** 9-(2,3-dihydroxypropoxy)-9-oxononanoic acid, **10*** (1R,6R,9R)-6,9,11-trihydroxy-4,7-megastigmadien-3-one 11-O-glucoside, **11*** 2,2′-(3-methylcyclohexane-1,1-diyl)diacetic acid, **12*** erythro-guaiacylglycerol-beta-O-4′-dehydrodisinapyl ether, **13*** (2*E*)-2-[2-(1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl)ethyl]-2-butenedioic acid, **14*** 8beta-(2-hydroxy-2-methyl-3-oxobutyryloxy)glucozaluzanin C, **15*** erythro-guaiacylglycerol-beta-O-4′-dehydrodisinapyl ether.

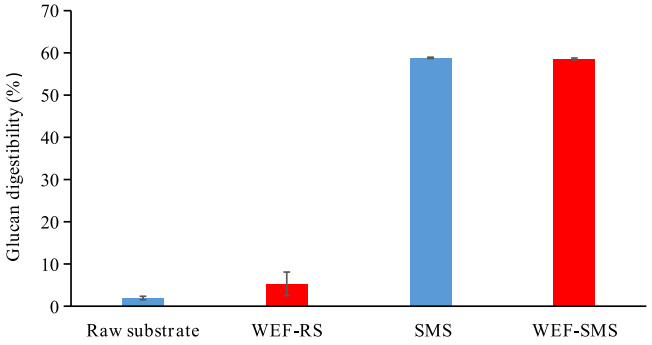

Fig. 5. Enzymatic digestibility of glucan contained in the raw substrate, WEF-raw substrate (WEF-RS), SMS, and WEF-SMS. Data refer to mean values ± standard error (SE).

Table 3
Chemical composition of the hydrolysates of SMS and WEF-SMS. Data refer to mean values ± standard error (SE).

Parameters	Units	Hydrolysates of	
		SMS	WEF-SMS
Glucose	g/L	16.5 ± 0.01	20.4 ± 0.04
Xylose	g/L	4.8 ± 0.00	5.6 ± 0.01
Levulinic acid	g/L	0.62 ± 0.00	0.51 ± 0.00
Formic acid	g/L	0.15 ± 0.00	0.05 ± 0.00
Acetic acid	g/L	ND	ND
Phenolic compounds	g/L	0.77 ± 0.01	0.42 ± 0.01
Total nitrogen	mg/L	858.4	435.4

ND, Not detected.

gluconolactone, ribonolactone, and caffeic acid 4-O-glucuronide), amino acid (including valine) and some peptide chains (including Leu-Gly-Phe, Ala-Phe, and Leu-Ser-Tyr) were found in the WEF-SMS hydrolysate (Fig. 6).

3.5. Fermentation of hydrolysates to ethanol

During the first 2.5 h of fermentation, approximately 45.9 % and 33.7 % of the initial glucose in the hydrolysate of SMS and WEF-SMS, respectively, was consumed by *S. cerevisiae* (Fig. 7a). This corresponds to volumetric consumption rates of 2.8 and 2.5 g/L h, respectively. In the case of WEF-SMS, a faster glucose consumption rate was observed between 2.5 and 5 h (3.8 g/L h), and the glucose was depleted soon after 5 h. The glucose in the SMS hydrolysate was depleted before 5 h, which is reasonable given that the lower initial amount of glucose required a shorter time for consumption by the yeast.

The SMS hydrolysate resulted in ethanol volumetric productivities of 1.04 g/L h during the first 2.5 h and 1.6 g/L h between 2.5 and 5 h (Fig. 7a). At five hours of fermentation, the ethanol concentration in the SMS hydrolysate reached its peak (6.5 g/ L), corresponding to a yield of 42.6 g/100 g glucose (Fig. 7b), after which it decreased due to evaporation. For the hydrolysate of WEF-SMS, 7.4 g/L ethanol (Fig. 7a), corresponding to a maximal yield of 39.6 g/100 g glucose (Fig. 7b), was achieved at 7.5 h. It was observed that the glucose consumption and ethanol formation were slower from 5 to 7.5 h compared to 0–5 h,

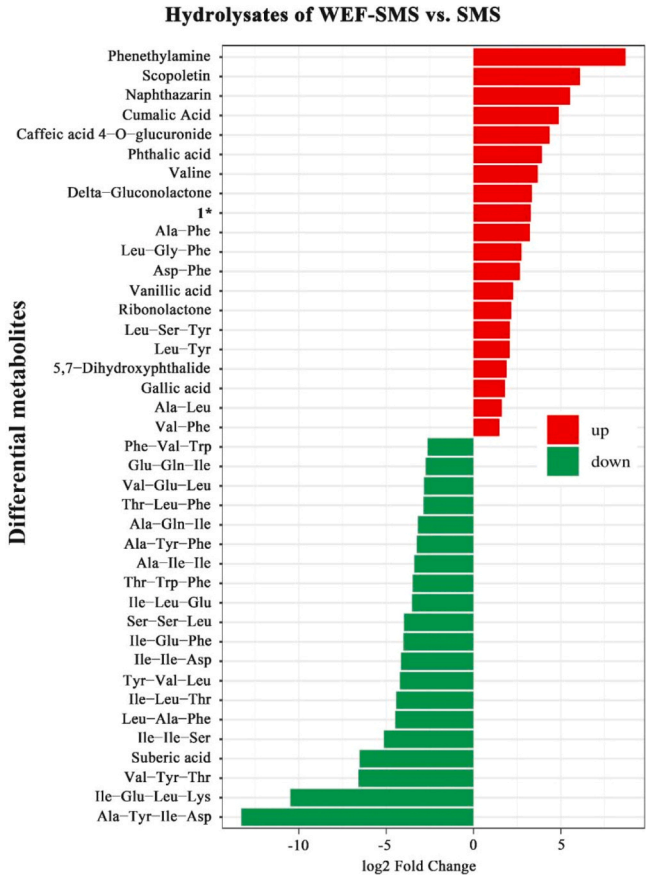

Fig. 6. The top 40 differential metabolites between the hydrolysates of WEF-SMS and SMS based on the log_2 (fold change) value. **1*** 2,4-dihydroxy-6-methoxy-3-formylacetophenone.

suggesting that fermentation was likely completed shortly after 5 h (Fig. 7a). A relatively higher ethanol yield for the WEF-SMS hydrolysate might be expected. However, compared with the volumetric productivity of ethanol in the fermentation of the SMS hydrolysate, the values for the fermentation of the WEF-SMS hydrolysates were notably lower, at 0.92 g/L h and 1.4 g/L h during the first and second 2.5 h, respectively (Fig. 7a).

4. Discussion

4.1. Shiitake pretreatment on hardwood

Our results of 42.6–47.6 % degradation of lignocellulosic compounds (Fig. 1) indicated that shiitake cultivation was a successful model for combined food production and hardwood pretreatment. Lignin, xylan, and glucan showed minor differences in degradation, which agrees with the previous studies (Lin et al., 2015; Yin et al., 2025). However, a selective degradation pattern in terms of the preferential removal of lignin and xylan (60–68 %), together with 70–80 % preservation of glucan was reported for shiitake (spawn M3790) cultivation on

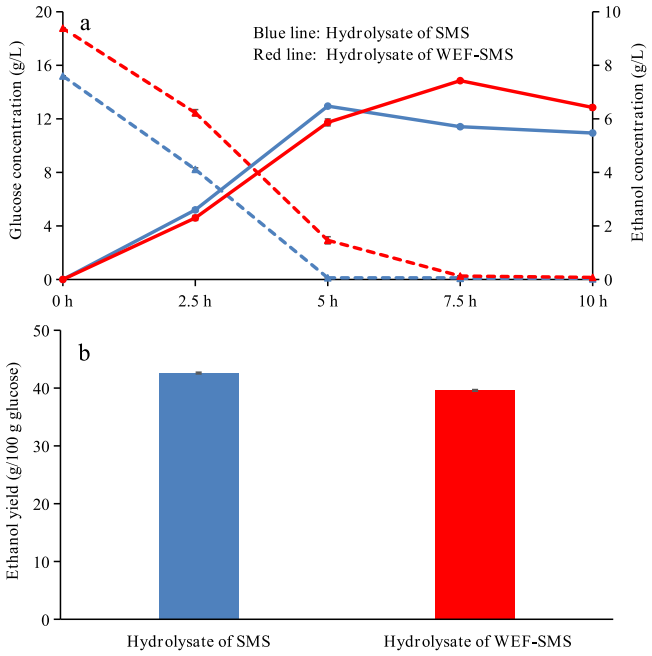

Fig. 7. Glucose consumption (dotted line) and ethanol production (solid line) (a) during *S. cerevisiae* fermentation of hydrolysates of SMS (blue) and WEF-SMS (red). Ethanol yield (b) during *S. cerevisiae* fermentation of hydrolysates of SMS and WEF-SMS. Data refer to mean values ± standard error (SE).

the substrates composed with single wood species (birch, alder, and aspen) (Chen et al., 2022a, 2022b). This shows that shiitake strains and substrate types are significant factors affecting the lignocellulose degradation.

The degradation of lignocellulosic compounds varied significantly with the cultivation stages; above 75 % of lignin and xylan degradation were happened during the first 63 days, and glucan degraded rapidly during the fruitification stage (Fig. 1). This finding is consistent with the growth behavior of shiitake that the fungus attack lignin and hemicelluloses for building its mycelium during the early vegetative growth stage, the broken cell wall structure then allow the uptake of cellulose for fructification completion (van Kuijk et al., 2015; Wu et al., 2024).

4.2. Formation of by-products in shiitake pretreated hardwood

Oxidoreductases and CAZymes secreted from fungi degrade lignocellulose into small molecules, those are subsequently serve as essential nutrients to support the growth of mycelium and fruit bodies (Floudas et al., 2012). The formation of numerous small molecule substances was evidenced by the accumulation of extractives in the growing substrates (Fig. 1). The mass of extractives in the substrate exhibited dynamic changes throughout the cultivation stages, showing a 51.5 % increase by Day 42 compared to that in the raw substrate; this was followed by a stabilization and a slight decrease by the end of shiitake cultivation (Fig. 1). Wei et al. (2020) reported a similar trend, considerable increase in extractives contents occurred during the mycelium colonization, after which the changes were smaller. The results further demonstrate an imbalance between the formation and utilization of small molecules. Although lignocellulose degradation occurred continuously during shiitake cultivation, producing various small molecules, these degradation products were predominantly utilized during the fructification stage.

The metabolite analysis presents the characteristics of small molecules and their dynamics over shiitake cultivation (Fig. 4). The top 20 up-regulated metabolites observed during the first 42 days represented the most abundant small molecules formed during the mycelium colonization stage (Fig. 4a), it is notable that none of these 20 molecules were subsequently down-regulated/utilized during the fructification stage (from day 42 to SMS, Fig. 4b), despite this stage is characterized by extensive utilization of small molecules (Fig. 1). Furthermore, the small molecules prominently formed during colonization stage (Fig. 4a) were no longer produced in significant quantities during the fructification stage (Fig. 4b). However, eleven of these compounds, including (L)-dehydroascorbic acid, triglochinic acid, and 5-hydroxy-2-methylchromone, exhibited significant accumulation when considering the entire cultivation process (Fig. 4c). The characteristics of the forming small molecules exhibited significant variation with lignocellulose degradation at different cultivation stages. Notably, the results suggest that shiitake displayed a distinct selective utilization pattern for these degradation products. Consequently, the non-utilized small molecules progressively accumulated in the SMS, forming shiitake pretreatment by-products.

The top 20 up-regulated metabolites in SMS versus raw substrate (Fig. 4c) presents the significant accumulated by-products from shiitake pretreatment, which could be classified as benzene and substituted derivatives, carboxylic acids, cinnamic acids and derivatives, flavonoids, organooxygen compounds, and benzopyrans (Table S2). Benzene is an important product of lignin valorization through various methodologies such as catalytic pyrolysis, hydrodeoxygenation, and combined catalytic processing (Liao et al., 2020; Meng et al., 2021). Cinnamic acids are key intermediates in the biosynthesis of lignin (Kim et al., 2013). Benzene and substituted derivatives, and cinnamic acids showed increased levels in the SMS compared to those in raw substrates (Fig. 4c), which might be the lignin degradation products in shiitake pretreatment. Lan et al. (2023) proposed a microbial valorization route for lignin to flavonoids. Starting from lignin degradation by ligninolytic microorganisms and the lignin-derived aromatic monomers as a platform compound, flavonoids can be produced using yeast or some bacteria via the biosynthesis pathway. The microbial environment in shiitake substrates (Eilertsen et al., 2023) may facilitate the formation of flavonoids (Fig. 4c). Carboxylic acids are typical by-products from the hydrolysis of carbohydrates (Jönsson and Martín, 2016; Martín et al., 2018). However, common aliphatic carboxylic acids from thermochemical pretreatment, such as acetic acid, formic acid, and levulinic acid, were not detected in the SMS (Fig. 4c), that is consistent with previous shiitake study (Chen et al., 2022b).

4.3. Potential effects of pretreatment by-products on downstream bioconversion processes

Water extraction effectively minimized the accumulation of by-products in the SMS and the resulting hydrolysate. Water extraction removed 46 % metabolite species belonging to the six most abundant chemical compound groups in the SMS (Fig. 2). Notably, among the top 20 accumulated by-products in the SMS (Fig. 4c), 10 of them (including 3-hydroxycoumarin, bergenin, and triglochinic acid) exhibited significant down-regulation in the WEF-SMS (Fig. 4d). Levulinic acid and formic acid were not found in the SMS, but detected in the hydrolysate with concentration of 0.62 and 0.15 g/L (Table 3). The formation of those can be attributed to the hydrolysis of carbohydrates by the used enzyme preparation (Cellic CTec 3). The absence of acetic acid in the hydrolysate suggests that Cellic CTec3 exhibited limited capability in hydrolyzing acetyl groups. The SMS hydrolysates contained also phenolic compounds with concentration of 0.77 g/L (Table 3), which were formed by lignin degradation and further ended up in the hydrolysates (Chen et al., 2022c). Short-chained carbohydrates and phenolics were detected in the extractives of SMS (Chen et al., 2022b; Wei et al., 2020). Compared to SMS hydrolysate, the above chemical compounds concentrations in WEF-SMS hydrolysate decreased by 17.8–66.7 % (Table 3).

The enzymatic digestibility of glucan contained in the SMS reached up to 58.8 % (Fig. 5), which was approximately 30 times higher than the

value achieved for raw substrate prior to cultivation. The trend confirms shiitake cultivation as a pretreatment method for enhancing the cellulose susceptible to enzymatic saccharification. However, this value is lower than those reported in previous shiitake studies, e.g., 80.3 % (Chen et al., 2022c), 86.7 % (Xiong et al., 2019) and 92.8 % (Chen et al., 2022a), these SMSs exhibiting a higher degradation of lignin (60–77 %) and hemicelluloses (57–70 %). The hydrolysis of WEF-SMS resulted in a comparable glucan digestibility of 58.6 % (Fig. 5). This finding suggests that the increased degradation of lignin and hemicelluloses play crucial roles in enhancing enzymatic saccharification efficiency, while the resulting degradation by-products exhibited negligible inhibitory effects on cellulolytic enzymes.

For the SMS hydrolysate, the ethanol yield corresponded to 83.4 % of the theoretical maximum yield (42.6 of 51.1 g/100 g glucose, Fig. 7b). The value is in good agreement with previously reported shiitake pretreatment studies (Chen et al., 2022c; Yin et al., 2025) and in the typical ranges achieved in different optimized thermochemical approaches, e.g., 80.4–89.7 % for dilute acid pretreated corn stover (Du et al., 2020; Qureshi et al., 2015; Yuan et al., 2022), 88.2 % for steam exploded corn stover combined with an immobilization technology (Kong et al., 2025), and 78 % for sequential dilute acid-alkali pretreated sugarcane bagasse (Hemansi and Saini, 2023). However, it is noteworthy that the WEF-SMS hydrolysate demonstrated lower volumetric productivity and yield compared to the SMS hydrolysate (39.6 vs. 42.6 g/100 g glucose, Fig. 7b), despite exhibiting reduced accumulation of by-products.

The sum total concentration of acetic acid, formic acid, and levulinic acid in SMS hydrolysate was 0.77 g/L (Table 3), the value was significantly lower than the levels typically observed in thermochemical hydrolysates (8.3–12.8 g/L) (Kong et al., 2025; Martín et al., 2018). and well below the theoretically inhibitory threshold of 100 mM (Larsson et al., 1999). The concentration of total phenolic compounds in the SMS hydrolysate, as determined by the Folin–Ciocalteu assay, was 0.77 g/L (Table 3), which falls within the range reported in previous shiitake studies (0.03–2 g/L; (Chen et al., 2022c; Yin et al., 2025)). It should be noted, however, that this value is considerably lower than those typically achieved using thermochemical processing methods (Martín et al., 2018). Although the potential inhibitory effects of distinctive by-products derived from shiitake pretreatment, such as (L)-dehydroascorbic acid and triglochinic acid, have not been thoroughly investigated, the considerable ethanol yield obtained from SMS hydrolysate (Fig. 7b) suggests that these compounds likely had minimal impact on fermentation performance. Consequently, mitigating fermentation inhibition by reducing the accumulation of by-products demonstrated negligible effectiveness (Fig. 7b).

During fermentation of cellulosic hydrolysates, nutrient supplementation is required for ensuring the metabolic activity of *S. cerevisiae* and avoiding sluggish fermentation (Chen et al., 2022c). Among nitrogen forms, amino acids serve as the main source of yeast assimilable nitrogen (Gobert et al., 2019). Fungal metabolism efficiently assimilates nitrogen from the substrate, leading to its concentration not only in the protein-rich fruiting bodies but particularly within the mycelial of SMS. Shiitake cultivation was found to significantly enhance the solubility of nitrogen sources present in the substrates (Chen et al., 2022a, 2022b). However, mycelia remaining in the SMS were dramatically removed during the water extraction process, resulting in significantly lower amounts of peptide (Fig. 6) and total nitrogen (Table 3) in the WEF-SMS hydrolysate. The lower nutritional richness of WEF-SMS hydrolysate contributed to the relatively poor fermentation performance (Fig. 7b). This hypothesis is supported by observations of *S. cerevisiae* cell growth during fermentation (Fig. S1). The cell density of *S. cerevisiae* in SMS hydrolysate increased rapidly from an initial value of 0.47, reaching a peak of 1.5 at 10 h, and subsequently remained stable. In contrast, in WEF-SMS hydrolysate, *S. cerevisiae* exhibited relatively slower growth during the early phase, reaching its maximum between 12.5 and 15 h. The observed lag phase in the growth curves is consistent with previous fermentation study under nutrient-limited conditions (Malairuang et al., 2020).

4.4. Future study on shiitake pretreatment

Shiitake is the most cultivated mushroom in the world, the approach of integrated production of protein-rich food and cellulosic ethanol using the lignocellulosic residues suits well the bio-based circular economy concept. In this study, enzymatic saccharification assays revealed that the digestibility of cellulose contained in the SMS reached up to 58.8 %, subsequently, the hydrolysates were fermented to ethanol yielding 42.6 g/100 g glucose. However, the glucose concentration in the hydrolysates of both SMS and WEF-SMS (ranging from 16.2 to 20.4 g/L) was considerably lower than that required by the currently recommended high gravity (HG) fermentation technology, which generally demands a glucose concentration exceeding 100 g/L in the media (Puligundla et al., 2019). The enzymatic digestibility of cellulose from the SMS represented the major limiting factor. In comparison with shiitake strain 212 and mixed hardwood chips used in this study, strain M3790 cultivated on single wood species (birch, alder, and aspen) achieved 60–68 % degradation of lignin and hemicelluloses, yielding a cellulose digestibility of the SMS as high as 92.8 % (Chen et al., 2022a, 2022c). A key focus of future research will be to enhance the degradation of lignin and hemicelluloses while maximizing cellulose preservation, through the targeted selection of shiitake strains and optimization of cultivation conditions. Meanwhile, recent studies have indicated that technologies including the implementation of controlled enzyme dosing and tailored feeding strategies could facilitates efficient high-solids enzymatic hydrolysis of lignocellulosic biomass (with final solids loading of 20–40 %) (da Silva et al., 2020; Qiao et al., 2024), thereby maximizing glucose concentration in the fermentation media. The applicability of this approach to SMS warrants further investigation.

Additionally, this study characterized the pretreatment by-products using the metabolite analysis and discussed their formation mechanism from the perspective of shiitake biological properties. Small molecules, including benzene, cinnamic acids, and flavonoids, were accumulated in the SMS as soluble extractives. As mentioned above, further enhancing the degradation of lignin and hemicelluloses represents a major focus in the shiitake pretreatment, which will inevitably lead to increased accumulation of extractives. Although this study, through a comparison of SMS and WEF-SMS, combined with the higher ethanol production potential demonstrated by SMS in previous research (Chen et al., 2022c), indicates that the inhibitory effects of by-products on downstream bioconversion processes are relatively limited, several aspects remain to be further clarified and explored in future studies. These include whether the types of by-products in SMS remain consistent under different degradation conditions, as well as their inhibition thresholds for enzymatic hydrolysis and fermentation.

5. Conclusion

Fungal pretreatment using shiitake effectively facilitated lignocellulose bioconversion. The characteristics of the formed metabolites exhibit significant variation with lignocellulose degradation at different cultivation stages. Shiitake mushroom demonstrated selective utilization of these degradation products during fructification, with non-utilized molecules accumulating in SMS extractives as pretreatment by-products. Water extraction prior to enzymatic saccharification effectively minimized the accumulation of by-products but did not result in improved saccharification and fermentation efficiencies compared to the base case without extraction. The inhibitory effects of shiitake pretreatment by-products on downstream biochemical processes were found to be limited.

CRediT authorship contribution statement

Feng Chen: Writing – review & editing, Writing – original draft,

Project administration, Funding acquisition, Conceptualization. **Jiale Xie:** Investigation. **Shengxu Qi:** Writing – original draft, Investigation. **Shaojun Xiong:** Writing – review & editing, Writing – original draft. **Carlos Martín:** Writing – review & editing, Writing – original draft.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.122270.

Data availability

Data will be made available on request.

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