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Shiitake cultivation on different lignocellulosic residues for food, feed and fuel – amino acids in fruit bodies and spent mushroom substrates

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ABSTRACT

This work evaluated the protein quality of shiitake mushroom cultivated on low-nitrogen wood substrates that has previously been shown to enhance biorefinery processes through biological pretreatment. The effects of different initial mushroom substrates (IMSs) on amino acid (AA) content in shiitake fruit bodies (FBs) and spent mushroom substrates (SMSs) were studied using alder, birch and aspen wood substrates with varying nitrogen levels by adding whey (0-2%). Twenty-two AAs, including nine essential AAs (EAAs), were detected and analyzed. Total AA (TAA) content reached up to 13% in FBs and 5% in SMSs. The FBs showed good protein quality according to FAO/WHO guidelines, despite a low nitrogen content (~0.6%) in IMS. Whey addition increased TAA and EAA levels in FBs while substrate species had distinct effects. Birch generally enhanced AA levels, alder reduced them, and aspen showed AA-specific but mostly positive responses. In SMS, whey addition also increased TAA, but the effects of wood species on TAA generally showed patterns opposite to that observed in FBs. Using 13 IMS chemical constituents as predictive variables in partial least squares regression, five models for AAs in FBs and 15 for SMS were achieved. The C/N ratio and soluble NO_2 were major predictors, whereas Klason lignin had the least influence. The results suggested that shiitake SMS is a valuable resource for potential protein extraction and biofuel production.

1. Introduction

Use of indigenous lignocellulosic low-grade residues (e.g. forest- or agro-based by-products and wastes) as substrates for healthy food production has several advantages: it adds value to industrial side streams and decreases dependency on imported food, which in turn reduces the need for long-distance transport associated with greenhouse gas emissions [1]. In this context, the cultivation of edible mushrooms, such as shiitake (*Lentinula edodes* (Berk.) Pegler), is particularly beneficial. This mushroom species grows especially well on lignocellulosic residues, such as leftovers from thinnings in young forests and logging residues or by-products like sawdust. The shiitake mushroom fruit bodies (FBs) are rich in vitamins (vitamin D_2, B_1, B_2, B_6, B_{12}, and niacin), minerals (potassium, manganese, magnesium, iron, and phosphorus) and other health-promoting ingredients associated with their immunomodulatory, antibacterial, cytostatic and antioxidant properties [2,3]. They can also be a source of plant-based protein, although not completely matching the protein quality of animal-based proteins [4,5]. However, when combined with other plant-based protein sources, for example within a balanced vegetarian diet, mushrooms can contribute to meeting nutritional protein requirement [5].

Recent research and development have indicated that the cultivation of edible fungi can be a crucial starting point of a cascade process to transform lignocellulosic biomass into valuable and diverse bio-based products [6]. By cultivating white-rot edible fungi (such as shiitake) using low value forest residues as the major growing substrate ingredients, the wood residues can be converted into food. During cultivation, the white-rot fungi secrete enzymes such as lignin- and manganese-peroxidases, and hemicellulases that can selectively degrade lignin and hemicellulose contained in the substrate. Thus, the wood substrate is transformed by the fungi into a cellulose-rich feedstock, known as spent mushroom substrate (SMS), suitable for production of, e.g., biofuels (ethanol, biogas, etc.) and biomaterials [6–10]. Therefore, the cultivation of edible fungi on wood substrate is

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functioning as a biological pretreatment of residual wood prior to biorefinery processes.

Depending on plant species, geographical origin and assortment of ingredients, different initial mushroom substrates (IMS) may affect biochemical characteristics of cultivated mushrooms [11]. Both the yield and nutritional composition, including protein content and quality, of FBs may vary. These differences can largely be attributed to variation in fungal responses to the chemical components and metabolic processes associated with different IMS, particularly in relation to lignocellulose degradation [12]. Metabolic byproducts formed during lignocellulose degradation may play distinct roles in mycelial growth and fruitification, thereby leading to variations in FB yield and protein contents, constituents and quality. Concomitantly, the substrate composition may change considerably during cultivation. Thus, a thorough understanding of the relationship between substrate composition and mushroom protein quality is essential to enable the production of edible mushroom FBs as a protein source and the utilization of SMS as a feedstock for value-added bio-based products. For both academic research and practical control of industrial mushroom production, key issues that remain to be investigated are how IMS species/formulation and which specific chemical components affect the protein content and constituents in FBs. Furthermore, fungal protein consists of numerous proteins built from various amino acids (AAs) [11–13], and individual AAs may be synthesized or converted through different interactions between IMS chemistry and fungal metabolic pathways. However, these relationships have not been extensively investigated in the existing literature.

In addition to the protein present in FBs, SMS may also represent a valuable protein source as it contains a considerable proportion of mycelia in addition to woody remnants [11]. However, the protein content, as well as the AA composition of these proteins in SMS, have not yet been clearly characterized. Addressing this knowledge gap is important for developing an efficient strategy to utilize SMS in a resource-efficient way. One scenario is that the proteins and/or AAs can be extracted from SMS prior to using the remaining lignocellulose as a cellulose-rich feedstock, e.g., for biobased products such as bioethanol.

Previous studies [7,8] have shown that a low nitrogen content in IMS enhances delignification of the lignocelluloses in the substrate and enrichment of cellulose in SMS, while still supporting a satisfactory mushroom yield. This can subsequently facilitate the bioconversion of SMS into cellulosic-based products such as bioethanol. However, the nitrogen level of growth media can affect edible fungi [14,15]. The consequences of a low nitrogen content of the IMS on protein content and AA composition of cultivated shiitake have rarely been reported. This is an important aspect for enabling a local, sustainable, and integrated supply of both food and biofuels.

The main aim of this study was to apply a D-optimal designed factorial experiment to study the protein content and AA profile in FBs and SMS of shiitake mushrooms, as influenced by nitrogen level and origin of the lignocellulosic substrate. Such results are essential for optimizing substrate formulations to support efficient mushroom-based food production and to maximize the value of SMS as a feedstock for biobased products. The dietary protein quality of FBs was evaluated according to FAO/WHO recommendations. Two stages of analysis were performed using partial least square (PLS) regression to examine the relationships between (i) AAs and substrate compositional species, and (ii) AAs and substrate chemical components (content of carbon, nitrogen, and lignocelluloses).

2. Materials and methods

2.1. Mushroom growing substrates

As lignocellulosic substrate models, sawdust (<4 mm) from white birch (*Betula pubescens* Ehrh.), alder (*Alnus incana* (L.) Moench) and aspen (*Populus tremula* L.) were used; all are hardwood species commonly found in boreal forests of the northern hemisphere. Wheat (*Triticum aestivum* L.) bran (≤2 mm), a residue of food production from the Swedish company Lantmännen, was added as a nutrient supplement. Whey powder (Whey-100, HSNG AB, Sweden), a by-product of cheese manufacture containing about 70% protein [8], was used as a nitrogen additive.

2.2. Experimental design and substrate preparation

A D-optimal experiment [16] with combined formula and quantitative factors was adopted for the design using MODDE 13.0 software (Sartorius AG, Sweden). The D-optimal design is generated by computer algorithms that select the most informative experimental runs while avoiding redundant runs often encountered in conventional and full factorial experiment. It offers an advantage of handling mixed factors within a single experiment, which is particularly suitable for present study that includes a non-quantitative factor – substrate species (alder, aspen and birch) – and an quantitative factor – percentage (%) of whey as nitrogen additions. The built-in robustness of the design allows for fitting linear, quadratic or even higher-order models, as well as performing statistical analyses.

The experiment comprised 17 runs and was designed based on blended hardwood sawdust (80% of dry weight, DW) and wheat bran (20% of DW) (Table 1). 'Sawdust from hardwood species' was a formulation factor and referred to the hardwood sawdust mass fraction of birch, alder and aspen. Addition of whey (0%, 1% and 2%) was a quantitative factor in the substrates and modeling. The design incorporated five replicated center points (N13-N17 in Table 1) using blends containing equal proportions of all wood species, with 1% whey. This experimental design allowed multivariate analysis and prediction of different outcomes from the experiment using PLS regression modeling, conducted using MODDE 13.0 software. Specific procedures were adopted according to Chen et al. [8].

Different initial mushroom substrates (IMSs) were prepared by mixing all ingredients according to the proportions listed in Table 1 and then adding water to adjust the moisture content of the substrates to 65% (wet based). The pH of the substrates was adjusted to approximately 6.36 by adding 1% $CaCO_3$ of the substrate DW. The preparation and performance of the mushroom cultivation experiment were carried out at the mushroom laboratory of the Swedish University of Agricultural Sciences in Umeå.

2.3. Mushroom cultivation and sampling

After blending all ingredients, 200 g of each IMS (with adjusted moisture and pH), was packed into separate transparent polypropylene micro-containers (125 × 65 × 80 mm, from Microsac). A lid equipped with micro-porous filters to allow gas exchanges was used to seal each micro-container. Five replicates of filled micro-containers for each treatment were pasteurized immediately at 85 °C in a hot-air oven for 4 h to prevent competitive microbial growth. After pasteurization, one of the five containers for each treatment was randomly chosen and collected for chemical analysis of the IMS after drying at 45 °C to constant weight (≥96 h) and milling to ≤ 0.5 mm.

Each pasteurized micro-container of IMS was inoculated with 5 g of shiitake (*Lentinula edodes* (Berk.) Pegler) spawn strain M3790 from Mycelia BVBA. The containers were then placed in a climate chamber for incubation, following the same procedures described in our previous publication [8].

Mushroom FBs per cultivation container were harvested manually and collected entirely, including small pieces. Only first flush was harvested. Upon harvesting, the whole block of the SMS in each container was also collected. The collected FBs and SMS per container were dried at 45 °C to constant weight (≥96 h) immediately after harvest and/or collection, then milled to ≤ 0.2 mm (Fritsch Pulverisette 14) and stored in airtight plastic bags at room temperature before further processing.

Table 1
Experimental design and fractions of substrate ingredients.

Treatment	Substrate ingredients (% DW)			Sawdust blend (proportion)			Ratio C/N
	Wheat bran	Sawdust	Whey	Birch	Alder	Aspen	(Actual)
N1	20	80	0	1	0	0	78.14
N2	20	80	0	0	1	0	67.82
N3	20	80	0	0	0	1	81.03
N4	20	80	0	0	0.5	0.5	67.46
N5	20	80	0	0.5	0	0.5	76.03
N6	20	80	0	0.5	0.5	0	73.35
N7	19.6	78.4	2	1	0	0	49.56
N8	19.6	78.4	2	0	1	0	43.41
N9	19.6	78.4	2	0	0	1	62.85
N10	19.6	78.4	2	0	0.5	0.5	51.98
N11	19.6	78.4	2	0.5	0	0.5	55.93
N12	19.6	78.4	2	0.5	0.5	0	51.60
N13	19.8	79.2	1	0.333	0.333	0.333	61.02
N14	19.8	79.2	1	0.333	0.333	0.333	64.33
N15	19.8	79.2	1	0.333	0.333	0.333	62.64
N16	19.8	79.2	1	0.333	0.333	0.333	62.07
N17	19.8	79.2	1	0.333	0.333	0.333	59.65

Table reproduced from Chen et al. [8]. DW, dry weight.

Before the analysis of AAs, FB or SMS powders from the four replicates of each treatment run were proportionally pooled (by 20% of each replicate's dry weight) and well mixed into one homogeneous sample.

2.4. Determination of nitrogen and lignocellulose contents

To investigate the relationship between AA content and initial substrate chemistry, 13 variables of the IMS constituents (carbon, nitrogen, and lignocelluloses) were analyzed. To determine soluble total nitrogen (TN), as well as NH_4^+-N, NO_3^--N and NO_2^--N, a water extraction was first performed. Of each sample for extraction, approximately 5 g dry mass (DM) was placed in a cotton cellulose thimble and extracted successively with approximately 100 mL of water for 6 h using a Soxhlet extractor (Buchi, B811) with a heating temperature at approximately 150 °C. When the reflux time was completed, the water extract was collected and diluted to 100 mL with distilled water. The collected samples of water extract were stored in a laboratory freezer at −20 °C until analysis by an accredited laboratory (EUROFINS, Sweden). The methodology for determining the content of total nitrogen (TN), total carbon (TC) and lignocellulosic components in the solid IMSs followed the same method detailed in a previous publication by Chen et al. [8].

2.5. Determination of crude protein and amino acids

The amounts of 22 free and bound AAs in each pooled SMS and FB samples of 17 treatments were quantified by UHPLC-QqQ-MSMS using AccQ•Tag™ derivatization (Waters, Milford, MA, USA). The analyses were conducted by the Swedish Metabolomics Centre (SMC) in Umeå, Sweden, following the SMC-protocol described in detail in the Supplementary Material.

Crude protein in FBs was calculated as total nitrogen (TN) × 4.38, according to Barros et al. [17] and Koutrotsios et al. [18].

2.6. Evaluation of protein quality

AA scores (in %) were used to evaluate the dietary protein quality of shiitake fruit bodies [19,20]. An EAA is considered as limiting AA when its score is less than 100%. The AA score was calculated using the following equation:

$$AA\ score\ (\%) = 100 \times \left[\frac{\text{mg EAA}_{fb}\ \left(\text{g protein}_{fb}\right)^{-1}}{\text{mg EAA}_{ref}\ (\text{g protein})^{-1}}\right] \quad (1)$$

where EAA_{fb} is the amount of essential AA (in mg) and $protein_{fb}$ (in g) is the amount of crude protein (TN × 4.38) in shiitake FBs. The ratio mg EAA_{ref} to g protein is the recommended AA score value from reference FAO/WHO [19].

2.7. Statistical analysis

Firstly, the influence of whey addition and IMS ingredient tree species on the content of each AA content (as the dependent response variable) was evaluated. This was analyzed using partial least squares (PLS) regression within the software MODDE 13.0 (Sartorius AG, Umea, Sweden).

Secondly, principal component analysis (PCA) using SIMCA 17.0 (Sartorius AG, Umea, Sweden) was performed to overview the data of 13 different IMS chemical constituents (contents of carbon, nitrogen and lignocelluloses) and the 22 AAs in FBs and SMS.

Thirdly, each of the 22 AAs in FBs and SMSs was considered as a dependent (***Y***) variable and then was modeled by PLS (in software SIMCA 17.0) using the 13 IMS chemical constituents as independent variables ($\boldsymbol{X_i}$) based on the following equation.

$$Y = \varphi + \sum_{i=1}^{13} \sigma_i X_i \quad (2)$$

where Y is the AA content, φ is a constant and σ_i is the coefficient for IMS chemical constituent X_i.

PLS modeling was used because co-linarites between variables could be handled within the software used. The number of PLS components and number of variables included in the final regression models were determined by optimizing Q^2 (total variation that can be predicted by the model) and minimizing *RMSE*cv (root mean square error of cross validation). The final optimization involved removing one of the independent variables at each time with the lowest VIP (variable importance in projection) value and coefficients of the model. The PLS model was considered valid when the prediction of the explained variation, Q^2, was larger than 0.4 (>40% of the total variation) and when the model error *RMSE*cv reached its local minimum.

3. Results

3.1. Mushroom production and recovery of SMS

The yield of mushroom FBs and recovery of SMS for the major treatments are presented in Supplementary Material Fig. S1. After a cultivation period of 74 – 98 days (depending on the treatment), FBs and

SMS were harvested simultaneously from each container. The yields of fresh FBs (the first flush only, with a moisture content of 90%) ranged from 475 to 762 g per kg of dry initial substrate, while the recovery of SMS was between 62 and 70% of the initial dry substrate mass. No significant differences were observed between the substrate species in either FB yields or SMS recovery. Whey addition at 1-2% significantly increased the FB yield ($p < 0.05$) but did not significantly affect SMS recovery (Fig. S1).

3.2. Profiles of amino acids

Tables 2 and 3 summarize the content (sum of bound and free) of each AA found in the FBs and SMS for each treatment. In total, 22 AAs were detected in the FBs and SMSs. Summing all 22 AAs, the total content of AAs (TAA) ranged from 89 to 129 mg per g of FBs (Table 2) and 27 to 51 mg per g of SMS (Table 3).

Among the 22 AAs, methionine (Met), glutamine (Gln) and leucine (Leu) were the top three most abundant. Conversely, histidine (His), tyrosine (Tyr), aspartic acid (Asp), and gamma-aminobutyric acid (GAB) had the lowest abundance in both examined materials.

Detected AAs were mostly abundant in bound form (Supplementary Fig. S2). Free AAs were in total at levels below 0.1% DM (average across all experimental runs) of SMS but 1.38% of FBs. As expected, the sum of free and bound AAs in FBs was at least two times higher than that in SMS. Fig. 1 shows the profiles of free, bound and free + bound AAs, highlighting the differences between free and bound AAs. Notably, Met was abundant in the bound form but negligible in the free form. Leu, threonine (Thr) and valine (Val) were also more abundant in bound than in free form. In contrast, His, lysine (Lys), Tyr, Asp, glutamic acid (Glu), and ornithine (Orn) existed almost only in the free form.

All nine EAAs (bound + free form) were present in the FBs and SMSs. The top four EAAs based on contents were Met, Leu, Val and Thr in both FB and SMS. Notably the contents of most EAAs, except for Met, in FBs were lower in comparison to previously published data ([21,22], also Supplementary Table S1). These differences can plausibly be attributed to variations in substrate compositions across different studies.

Table 4 shows AA scores calculated using Eq. (1) based on data of EAAs in FB protein and the corresponding FAO/WHO reference pattern. Five out of the nine EAAs in FBs were comparable to or surpassed the recommended AA reference scores in protein defined by WHO/FAO/UNU in 2007 [20] and reinforced by FAO/WHO in 2013 [19]. This was true even for the treatments without whey addition. Four other EAAs (His, isoleucine (Ile), Lys and phenylalanine (Phe) + Tyr) did not meet the recommended criterion because their scores were far below 100%. Notably, the birch and aspen substrates resulted in slightly higher AA scores than alder, and AA scores increased when more nitrogen was available in the IMS, i.e., when whey was added.

Furthermore, according to data in WHO/FAO/UNU 2007 [20], the sum of EAA requirement is 277 mg per g of dietary protein. In the present study, the sum of required EAAs in shiitake FBs was 313 and 394 mg g^{-1} protein for birch substrate (0 and 2% whey, respectively), 248 and 263 mg g^{-1} protein for alder substrate (0 and 2% whey, respectively) and 310 and 366 mg g^{-1} protein for aspen substrate (0 and 2% whey, respectively) (Table 4). These results confirm the good food protein quality of shiitake mushrooms, in agreement with reports by other researchers [21–23].

3.3. Effects of N addition and substrate species on AA contents

Fig. 2 shows the main effect plots (Fig. 2A1 and 2B1) and contour plots (Fig. 2A2-4 and 2B2-4) resulting from significant PLS regression models for TAA (statistical data shown in Tables 1 and 2). It was evident that whey addition imposed a significant and major positive effect on TAA as well as most individual AAs, except for a few observations, in FBs and SMS (Fig. 2A1 and 2B1). Tree species of the substrate was also influential but less significant. This is visualized by the ternary contour pattern of TAA in relation to both whey addition and tree species fractions (Fig. 2A2-4 and 2B2-4). The ternary contour comprises three axes, each representing the fraction (0 – 1) of one tree species in the IMS. A fraction of 1 (at each corner) corresponds to a pure ingredient of a tree species, whereas 0 means it is excluded in IMS. The center point of each contour plot represents an equal mixture (one-third each) the three tree species in the IMS. As exhibited in the contours, TAA in FBs was positively and linearly correlated with birch and aspen, but negatively with alder (Fig. 2A2-4). However, TAA in SMS showed a non-linear relation (Fig. 2B2-4) due to interaction between birch and alder. The highest TAA contents in SMS were associated with an increasing proportion of alder but decreasing proportion of birch, whereas the lowest TAA contents were in a pure aspen fraction (Fig. 2B2-4). These differences in TAA may reflect recalcitrance of the tested hardwood substrates against fungal decay of the wood carbon matrices and associated metabolic activities. Notably, PLS models for AA content in SMS showed higher significant levels than those in FBs (Table 2 vs. 3).

A similar phenomenon was observed for individual EAAs in shiitake FBs (Fig. 3A and B). In general, as illustrated in Fig. 3A and B, whey addition had a positive correlation with the contents of all EAAs. This was also the case for the birch fraction, but alder had a negative correlation to the contents of EAAs in FBs. Interestingly, the role of aspen was positive in four of nine EAAs (Ile, Met, Phe and Val) but negative in the remaining five EAAs (Fig. 3B).

3.4. Correlation between substrates chemical constituents and AA content

The results from determination of 13 chemical constituents in the IMSs are shown in Supplementary Table S2, including total carbon, different forms of soluble nitrogen and lignocelluloses (lignin, cellulose and hemicellulose). These data were used to investigate correlations between AAs and substrate chemistry in order to gain a deeper understanding of the relationship between AAs and substrate ingredient species and whey addition. The analysis was carried out in two steps, as presented below.

3.4.1. Principal components analysis (PCA) to overview multivariate pattern

Principal components analysis (PCA) was first used to overview the data and gain general insights into correlations between substrate composition and AA content. The dataset included 17 observations each for FBs and SMSs, respectively, and 13 variables of IMS chemical constituents (Supplementary Table S2) and 22 variables of AAs, forming a 34 x 35 data matrix.

The two first principal components (PC1 and PC2), shown in the biplot in Fig. 4, explained 57.5 and 15.5%, respectively, of the total variance. The variation in PC1 was explained by AA contents and all AAs (except Ile) were clustered in the far right. PC1 separated the objects of SMSs (SMS1-SMS17) and FBs (FB1-FB17) from left to right along this dimension due to their differences in AA content, with the objects of mushroom FBs close to most AAs. The PC2 dimension was largely driven by the ratio of carbon to nitrogen (TC/TN, in down space) and total nitrogen (TN, upper space), also representing a complex nitrogen gradient from low to high. Thus, PC1 represented the AAs in FBs and SMSs, whereas PC2 represented the chemical composition of IMSs.

Along PC2, the objects SMS1-SMS6 and FB1-FB6 without whey were clearly separated from SMS7-SMS17 and FB7-FB17 with 1-2% whey. It was noticeable that FB7-FB12 (2% whey) and FB13-FB17 (1% whey) were further grouped separately, in contrast to SMS7-SMS17, which formed mostly one group. The contents of acid soluble lignin and Klason lignin in the initial substrate were grouped together with total carbon (TC) in the upper space along PC2, while the cellulose, hemicellulose and ethanol extractives were in another group in the lower space. This showed that these groups were negatively correlated. Interestingly, the lignin group seemed to be more positively related to objects with whey addition, separating from the other group that was distributed closer to

Table 2
Content (mg g-1 DM) of amino acids (sum of bound and free) in shiitake fruit bodies.

Factor		Essential									Conditionally essential					Non-essential					Others		
Whey	Wood [a]	His [b]	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Arg	Gln	Gly	Pro	Tyr	Ala	Asn	Asp	Glu	Ser	Cit	GAB	Orn
%	B/Al/As																						
0	1/0/0	0.17	0.27	7.08	0.70	11.19	2.36	5.72	3.45	4.99	5.60	14.14	4.70	4.69	0.20	5.29	5.04	0.29	0.84	6.02	6.04	0.30	0.49
	0/1/0	0.19	0.27	7.31	0.81	11.61	2.37	5.77	3.34	4.92	5.32	13.81	4.42	4.66	0.26	6.05	5.13	0.32	0.92	6.45	5.80	0.30	0.48
	0/0/1	0.17	0.25	7.13	0.71	12.68	2.40	5.80	3.05	5.83	5.58	14.11	4.60	4.81	0.24	5.67	4.88	0.37	0.71	4.89	4.70	0.21	0.67
	0/0.5/0.5	0.18	0.39	7.15	0.78	11.23	2.43	6.45	3.38	5.15	5.69	13.22	4.62	4.83	0.31	5.99	5.12	0.36	0.77	6.44	5.12	0.32	0.58
	0.5/0/0.5	0.15	0.35	7.34	0.66	11.97	2.52	6.17	3.96	5.76	5.84	13.20	5.25	5.27	0.28	6.16	5.31	0.33	0.92	6.60	6.31	0.30	0.54
	0.5/0.5/0	0.21	0.43	8.09	1.15	12.93	2.71	6.82	3.76	5.97	6.03	14.64	4.68	5.26	0.34	6.12	5.58	0.31	0.93	5.50	5.51	0.32	1.29
1	0.3/0.3/0.3	0.23 ± 0.02	0.39 ± 0.03	7.63 ± 0.25	1.00 ± 0.08	12.71 ± 0.59	2.62 ± 0.10	6.62 ± 0.44	3.73 ± 0.24	5.33 ± 0.12	6.09 ± 0.30	14.96 ± 0.59	4.70 ± 0.22	4.94 ± 0.17	0.23 ± 0.01	5.98 ± 0.01	5.46 ± 0.27	0.34 ± 0.20	1.06 ± 0.05	6.18 ± 0.34	5.78 ± 0.36	0.30 ± 0.02	0.96 ± 0.18
2	1/0/0	0.34	0.58	9.71	1.75	16.31	3.66	8.26	5.02	6.67	8.12	19.75	5.49	6.29	0.33	7.47	7.65	0.32	1.15	6.98	9.30	0.55	1.70
	0/1/0	0.27	0.43	7.48	1.22	12.30	2.83	7.17	4.50	5.55	6.74	14.37	4.57	5.26	0.30	6.71	5.84	0.24	1.10	6.62	7.12	0.45	0.80
	0/0/1	0.31	0.61	10.06	1.30	16.11	3.62	8.16	4.69	7.43	8.44	18.78	5.87	7.02	0.12	7.86	7.56	0.28	0.95	7.48	8.47	0.64	1.30
	0/0.5/0.5	0.27	0.53	9.32	1.48	14.97	3.26	7.45	4.22	6.39	7.95	17.73	4.92	5.31	0.29	7.05	6.83	0.32	1.13	6.44	7.24	0.58	1.70
	0.5/0/0.5	0.37	0.60	8.50	1.76	13.45	3.58	7.98	4.95	6.25	7.68	16.04	5.26	6.09	0.36	6.87	6.37	0.30	1.22	8.42	6.61	0.61	1.75
	0.5/0.5/0	0.24	0.36	10.47	1.54	16.37	2.53	7.46	3.71	6.90	6.02	18.10	4.93	6.00	0.20	5.80	6.92	0.37	1.21	4.69	6.32	0.39	1.63
No. observ.		17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17
d.f.		13	13	11	11	10	11	12	12	12	11	10	12	11	11	10	11	9	11	12	11	12	12
Q^2		0.44	0.12	0.15	0.41	0.18	0.36	0.21	0.26	0.48	0.37	0.33	0.19	0.28	−0.20	0.08	0.31	0.11	0.65	0.11	0.20	0.51	0.24
R^2		0.69	0.58	0.70	0.79	0.69	0.70	0.59	0.56	0.83	0.71	0.81	0.52	0.70	0.40	0.53	0.78	0.47	0.80	0.29	0.68	0.82	0.61
R^2adj		0.62	0.48	0.56	0.72	0.51	0.60	0.49	0.45	0.72	0.62	0.70	0.36	0.61	0.13	0.38	0.68	−0.06	0.71	0.05	0.53	0.76	0.48
Signif. PLS		***	**	*	***	*	**	**	*	**	**	**	*	**	ns	*	**	ns	***	ns	*	***	*

[a] B/Al/As: Birch/Alder/Aspen; [b] Abbreviations for amino acids: His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Thr = threonine, Trp = tryptophan, Val = valine, Arg = arginine, Gln = glutamine, Gly = glycine, Pro = proline, Tyr = tyrosine, Ala = alanine, Asn = asparagine, Asp = aspartic acid, Glu = glutamic acid, Ser = serine, Cit = citrulline, GAB = gamma-aminobutyric acid, Orn = ornithine. Significance level of PLS regression: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant. DM: dry mass.

Table 3
Content (mg g-1 DM) of amino acids (sum of bound and free) in shiitake spent substrates (SMS).

Factor		Essential									Conditionally essential					Non-essential					Others		
Whey	Wood	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Arg	Gln	Gly	Pro	Tyr	Ala	Asn	Asp	Glu	Ser	Cit	GAB	Orn
%	B/Al/As																						
0	1/0/0	0.02	0.33	3.55	0.09	4.76	0.69	2.08	0.72	2.50	1.78	3.55	2.08	1.82	0.04	2.91	1.92	0.04	0.07	1.90	1.47	0.05	0.07
	0/1/0	0.02	0.68	3.56	0.09	4.68	0.70	2.40	0.69	2.78	1.77	3.96	2.09	2.12	0.04	2.82	1.98	0.04	0.04	2.38	1.57	0.05	0.05
	0/0/1	0.03	0.17	3.03	0.07	3.57	0.58	2.02	0.59	2.18	1.64	3.48	1.77	1.67	0.03	2.09	1.62	0.05	0.07	2.54	1.35	0.06	0.03
	0/0.5/0.5	0.02	0.51	3.31	0.11	4.07	0.60	2.25	0.74	2.33	1.67	3.47	1.96	2.16	0.04	2.29	1.81	0.05	0.08	1.88	1.40	0.08	0.05
	0.5/0/0.5	0.02	0.23	3.08	0.09	3.44	0.56	2.05	0.70	1.90	1.49	2.93	1.88	1.68	0.03	2.34	1.62	0.04	0.08	1.55	1.39	0.06	0.06
	0.5/0.5/0	0.02	0.53	3.17	0.08	4.13	0.68	2.42	0.67	2.33	1.77	3.68	1.95	2.13	0.04	2.37	1.79	0.04	0.06	2.25	1.56	0.07	0.05
1	0.3/0.3/0.3	0.03 ± 0.00	0.44 ± 0.02	3.98 ± 0.14	0.10 ± 0.01	5.31 ± 0.06	0.75 ± 0.03	2.75 ± 0.14	0.86 ± 0.06	2.70 ± 0.13	2.05 ± 0.11	4.36 ± 0.17	2.32 ± 0.15	2.34 ± 0.17	0.04 ± 0.00	2.95 ± 0.12	2.25 ± 0.11	0.04 ± 0.00	0.07 ± 0.01	2.46 ± 0.17	1.74 ± 0.08	0.06 ± 0.00	0.06 ± 0.00
2	1/0/0	0.03	0.32	5.27	0.13	6.82	1.05	3.24	1.32	3.79	2.86	6.43	3.07	2.94	0.04	4.05	3.02	0.08	0.11	3.41	2.04	0.07	0.16
	0/1/0	0.03	0.85	5.03	0.12	7.57	1.04	3.55	1.13	3.51	2.96	6.14	2.89	3.32	0.04	3.48	3.11	0.05	0.07	3.67	2.34	0.09	0.06
	0/0/1	0.03	0.25	4.31	0.14	5.31	0.72	2.51	0.88	2.92	2.28	3.94	2.20	2.52	0.04	3.00	2.31	0.06	0.08	2.16	1.61	0.07	0.08
	0/0.5/0.5	0.03	0.52	4.43	0.09	6.04	0.92	3.14	1.01	3.31	2.29	5.11	2.61	2.96	0.04	3.52	2.54	0.06	0.08	2.76	2.03	0.09	0.05
	0.5/0/0.5	0.03	0.36	4.30	0.15	6.00	0.88	2.85	1.01	2.98	2.35	4.30	2.74	2.62	0.05	3.30	2.42	0.06	0.09	2.42	2.16	0.08	0.08
	0.5/0.5/0	0.03	0.50	4.52	0.10	6.22	1.05	3.25	1.18	3.22	2.70	5.66	2.70	2.77	0.04	3.24	2.67	0.05	0.08	2.84	2.03	0.08	0.07
No. observ.		17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17
d.f.		12	10	9	12	10	9	10	10	10	11	9	11	12	9	9	10	10	11	11	10	11	12
Q^2		0.39	0.85	0.70	0.20	0.75	0.73	0.66	0.38	0.65	0.64	0.50	0.53	0.57	0.03	0.72	0.66	0.21	0.28	0.15	0.60	0.17	0.40
R^2		0.52	0.92	0.94	0.49	0.93	0.93	0.85	0.84	0.89	0.86	0.91	0.79	0.80	0.42	0.91	0.90	0.76	0.57	0.59	0.84	0.66	0.66
R^2adj		0.41	0.87	0.89	0.36	0.89	0.88	0.78	0.76	0.83	0.81	0.85	0.71	0.75	0.04	0.85	0.85	0.64	0.42	0.45	0.77	0.53	0.57
Signif. PLS		*	***	***	*	***	***	**	**	***	***	***	***	***	ns	**	***	**	*	*	**	*	**

Full names of sawdust species and amino acids can be found in footnotes of Table 2. DM: dry mass.

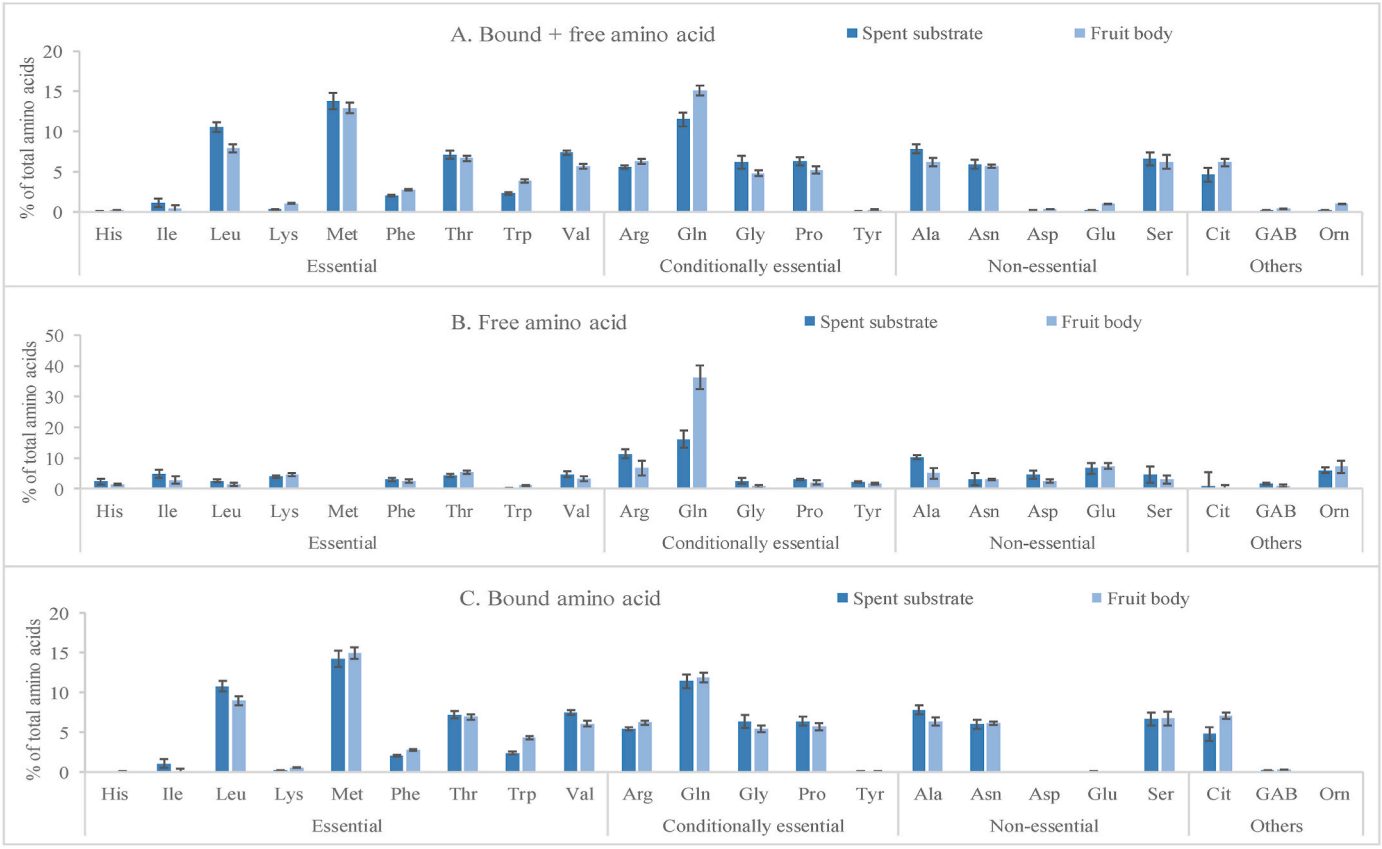

Fig. 1. Relative abundance of different amino acids (AA) in total sum of all AAs across all treatments.

Table 4
Essential amino acids (EAA) in protein of shiitake mushroom fruit bodies (FB) and their AA scores (%)[a], according to the recommended reference pattern by FAO/WHO [19], under various substrate conditions.

	Whey	His	Ile	Leu	Lys	Met + Cys [b]	Phe + Tyr	Thr	Trp	Val	Sum of EAAs
Reference pattern FAO/WHO (2013) [mg EAA_{ref} (g protein)$^{-1}$]		15	30	59	45	22	38	23	6	39	277
EAAs in FBs [mg (g protein)$^{-1}$] this study											
Birch	0%	1.51	2.34	61.28	6.07	96.80	22.16	49.51	29.80	43.13	313
Alder	0%	1.27	1.85	49.25	5.44	78.17	17.64	38.86	22.47	33.10	248
Aspen	0%	1.36	2.00	57.75	5.77	102.71	21.46	46.99	24.69	47.21	310
Birch	2%	2.52	4.34	72.67	13.12	122.11	29.87	61.84	37.54	49.95	394
Alder	2%	1.71	2.69	46.77	7.65	76.92	19.57	44.82	28.11	34.69	263
Aspen	2%	2.15	4.23	70.22	9.10	112.50	26.12	56.95	32.72	51.92	366
Amino acid score (%)											
Birch	0%	10	8	104	15	440	58	215	497	111	
Alder	0%	8	6	84	12	355	46	169	375	85	
Aspen	0%	9	7	98	13	578	57	204	412	121	
Mean (SE)	0%	9 (1)	7 (1)	96 (11)	13 (2)	458 (113)	54 (7)	196 (24)	428 (63)	106 (19)	
Birch	2%	17	14	123	29	550	79	269	626	128	
Alder	2%	11	9	79	17	350	52	195	469	89	
Aspen	2%	14	14	119	20	511	69	248	545	133	
Mean (SE)	2%	14 (3)	12 (3)	107 (24)	22 (6)	470 (106)	67 (14)	237 (38)	547 (79)	117 (24)	

[a] AA score (%) calculated using Eq. (1) (see section 2.6).
[b] Cys (cysteine) was not detected. SE: standard error within parenthesis.

the objects without whey treatment, especially FB1-FB6. It was also notable that soluble NO_2 (down and near TC/TN) was negatively

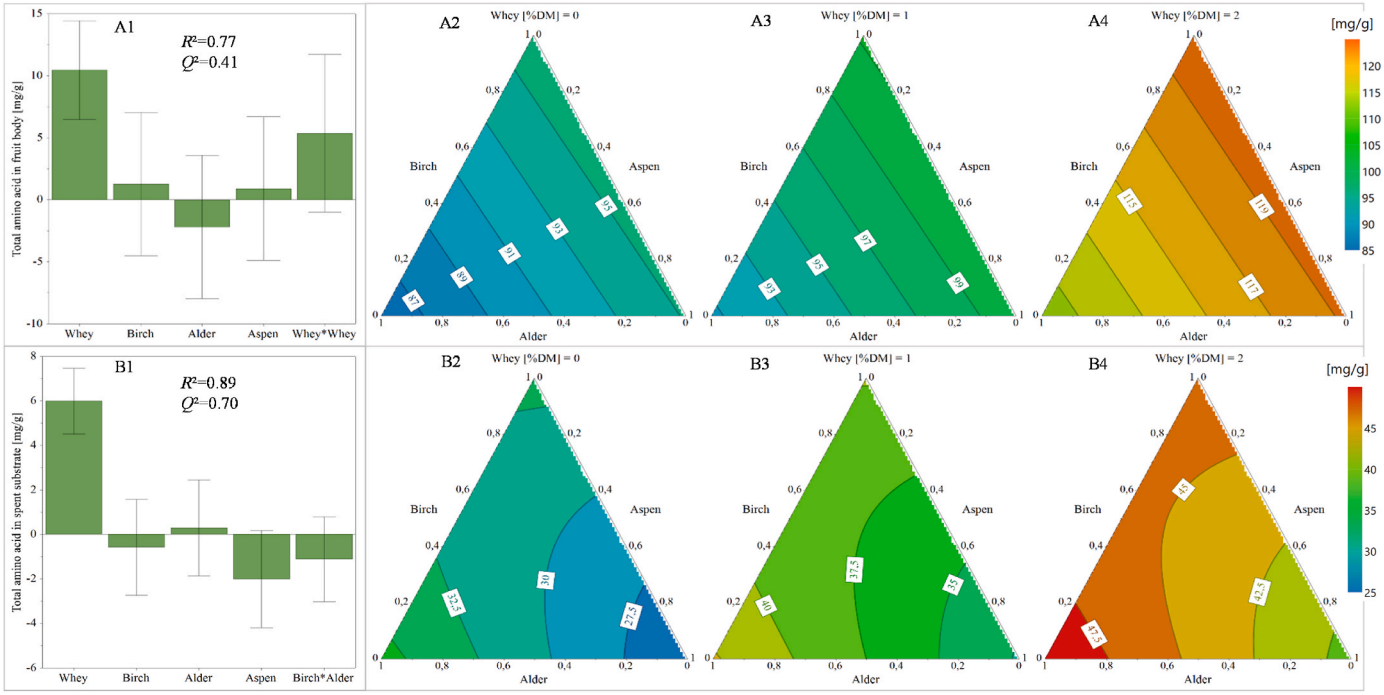

Fig. 2. Main effect plots for scaled and centralized factors along with response contour plots predicted with PLS models for the total content (TAA) of all 22 amino acids in fruit bodies (A) and spent substrate (B). Bars in plots (A1, and B1) refer to 95% confidence level. In each of the ternary contour plots, the axes represent the fraction (0 – 1) of each tree species (birch, alder and aspen) in the initial mushroom substrate (IMS). A fraction of 1 (at each corner) corresponds to a pure ingredient of a single tree species, whereas 0 indicates that the tree species was excluded in the IMS. The center point of each contour plot represents an equal mixture (one-third each) of the three tree species in the IMS. The colors gradient from red to blue in response contour plots A2 - A4 and B2 - B4 indicate the TAA content (mg g^{-1}), corresponding to the values shown in the labels and legend bar. Further explanations are provided in Section 3.3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

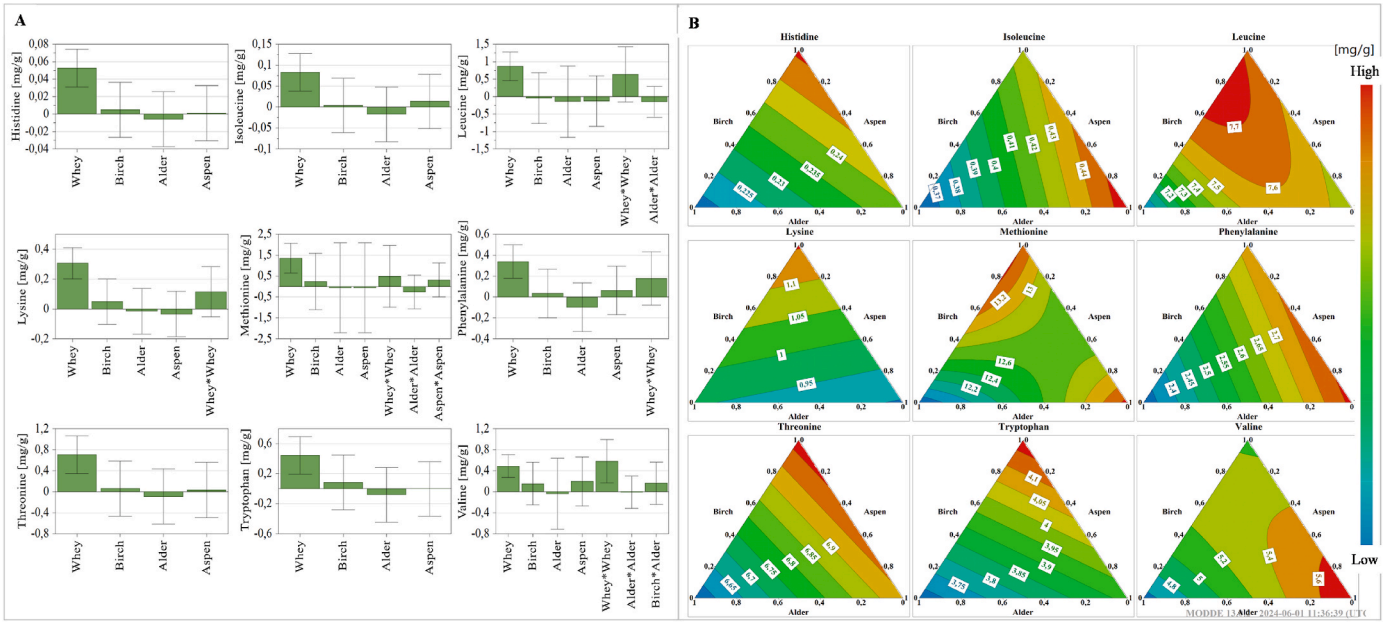

Fig. 3. Main effect plots for scaled and centralized factors along with response contour plots predicted with PLS models for the content of nine essential amino acids (EAA) in fruit bodies. Bars in plots A refer to 95% confidence level. The colour gradient from red to blue in contour plots B indicates the EAA content (mg g^{-1}) at 1% whey addition (i.e., the average level), corresponding to the values shown in the labels and legend bar, from high to low. Further details are provided in the caption of Fig. 2 and in Section 3.3 of the main text. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

correlated to soluble NH_4 and NO_3 forming one group (upwards) together with TN and water extractive.

3.4.2. PLS regression models to predict correlations

To gain further insights, PLS regression was performed to model the influence of the initial chemical substrate components (X_i in Eq. (2)) on single AAs (Y) in FBs or SMSs. The valid models, qualified in terms of the

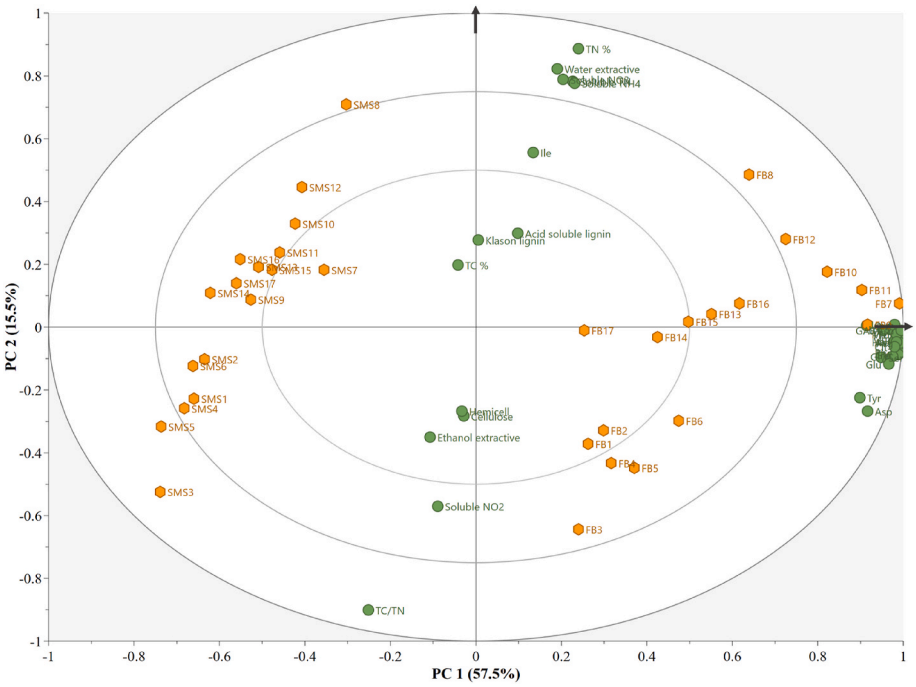

Fig. 4. Biplot for PCA analysis of 22 amino acids in fruit bodies (FB1-17) and SMS (SMS1-17) and the 13 components of the initial mushroom substrates. Four principal components were included in the PCA model explaining 86.9 % with a prediction value (Q^2) of 70% of the total variation.

parameters R^2x, R^2y and especially Q^2, are shown in Table 5 and expressed following Eq. (2). Five AAs contained in FBs (two EAAs) and 15 AAs in SMS (seven EAAs) were successfully regressed with IMS chemical components. Less valid PLS regression models were achieved for AAs in FB than those in SMS, suggesting a larger influence of substrate components on the contents of AAs in SMS.

Among the 13 variables of initial substrate constituents, nitrogen associated variables influenced more models than lignocellulosic variables (Table 5). Total nitrogen (TN) was positively correlated but total carbon (TC) and the ratio of TC/TN negatively correlated to the AA contents in whichever model they entered. All valid models included the variable soluble NO_2, which exhibited a positive effect on the contents of FB AAs but a negative effect on SMS AAs. Soluble NH_4 and NO_3 showed mostly positive coefficients for AAs, except for a couple of cases, in both FB and SMS related models.

Regarding the variables of lignocellulosic components, the acid soluble lignin entered all five models for FBs and 9 of 15 models for SMS. Hemicellulose and cellulose were second and third to acid soluble lignin and entered 4 vs. 3 models for FB and 6 vs. 8 for SMS, respectively. Notably, the coefficients of hemicellulose were positive not only for FB-AAs, which was same as acid soluble lignin and cellulose, but also for SMS-AAs. The coefficients of cellulose were mostly negative in SMS models, where acid soluble lignin was found to be mostly positive. In only three models, Klason lignin was included, making it the least "favorable" independent variable that entered PLS regressions compared to other substrate component variables.

Interestingly, four of five predictable AAs in FBs were found in free form only (c.f. Fig. 1), whereas 15 AAs in the PLS models for SMS appeared mostly in bound form.

4. Discussion

4.1. Shiitake cultivation – a resource efficient route to produce plant-based protein

This study demonstrated that both shiitake FBs and SMS contained 22 different AAs, including all nine EAAs that people need to obtain through their diet. This was true regardless of variations in the initial substrate ingredients/species and/or composition, including nitrogen content. Shiitake FBs, even when grown on low nitrogen substrates from forest-based residues and byproducts, had a generally good AA profile for humans according to both WHO/FAO/UNU 2007 [20] and FAO/WHO 2013 [19] (Table 4). Further, the proteins and AAs in SMS offer considerable value for human food, in addition to animal feed formulations [24]. All these facts indicate that the production of shiitake-based protein can be used to initiate a cascade process (Fig. 5), where lignocellulosic biomass is transformed into valuable biobased products (e.g., bioethanol), leading to enhanced resource efficiency. However, one challenge of a completely plant-based diet is the deficiency of specific EAA in certain plant protein sources [5]. Plant-based proteins are typically lower in Met compared with animal-based proteins [25]. Interestingly, our cultivated shiitake FBs contained all nine EAAs, with Met and Trp levels surpassing the reference criterion of FAO/WHO 2013 [19] (Table 4). The Met content, approximately 2% of the protein content, was comparable to the Met content in animal-based proteins like milk and casein [25]. Therefore, cultivated shiitake can be used as a natural part of a sustainable, well-balanced plant-based diet.

As our analyses showed (Table 2; Fig. 1B), shiitake FBs contained considerable amount of Glu that in its free form generates umami taste. Umami, described as a savory and satisfying taste, can partially enhance appetite and compensate for the loss of smell. This adds more value to the cultivation of shiitake as a food source.

4.2. Effects of substrate composition on AA profile and content

The results demonstrated that the addition of whey had a greater influence than hardwood ingredient species on the AA content. By adding 2% whey protein to the initial wood substrate, levels of AAs increased considerably (Tables 2 and 3). It is understandable that wood ingredients have a low content of AAs and/or other nitrogen sources, whereas whey can be either a direct source of AAs or source of nitrogen for AA synthesis. The added whey may have been physically adsorbed to surfaces of the wood particles or dissolved in the water solution contained in the substrate. Fungi may either directly uptake an AA or

Table 5
Partial least squares regression (PLS) models for amino acids (bound + free) in fruit bodies and spent substrates of shiitake mushroom.

Y	About model [a]					Coefficient σ_i for X_i-variable (initial substrate components)												
Amino acid [b]	No of PC	R^2_X	R^2_Y	Q^2	*RMSEcv*	Constant φ	TN	TC	TC/TN	Solu-ble TN	Solu-ble NH_4	Solu-ble NO_3	Solu-ble NO_2	Cellu-lose	Hemi-cellu-lose	Klason lignin	Acid solub. lignin	Water extractive	Etha-nol extractive
Fruit body																			
His	4	0.965	0.721	0.436	0.046	3.742			−0.287	0.335	0.249		0.431				0.557		
Lys	3	0.842	0.865	0.601	0.236	3.047	0.359		−0.456			0.307	0.377	0.327	0.515		0.441		
Glu	4	0.899	0.835	0.572	0.097	6.386	0.170		−0.354		−0.108	0.635	0.084	0.041	0.406		0.171		
GAB	3	0.777	0.846	0.433	0.105	2.874		−0.469	−0.327	0.295	0.352		0.287		0.433		0.235		
Orn	4	0.877	0.830	0.567	0.304	2.146			−0.494			0.327	0.296	0.584	0.415		0.639		−0.192
Spent substrate (SMS)																			
Ile	4	0.863	0.924	0.755	0.081	2.598	0.017	0.193	0.002	−0.130	0.124	0.075	−0.178			0.668	−0.184	0.207	
Leu	4	0.892	0.941	0.813	0.255	5.977	0.395	−0.315	−0.408	0.191			−0.029	−0.117			0.300		0.303
Met	4	0.900	0.925	0.836	0.429	4.647	0.342	−0.086	−0.290	−0.017	0.316		−0.062	−0.093			0.107	0.013	
Phe	4	0.843	0.914	0.716	0.080	4.86	0.528	−0.214	−0.419	−0.298	0.161	0.092	−0.031		0.083		0.190		
Thr	4	0.851	0.918	0.768	0.205	5.647	0.385		−0.367	−0.302	0.212	0.276	−0.147				−0.062	−0.034	−0.071
Trp	4	0.876	0.941	0.824	0.082	4.241	0.590	−0.339	−0.633	−0.416	0.187		0.204	−0.434		−0.367			
Val	4	0.800	0.902	0.563	0.292	5.504	0.426	−0.406	−0.483	−0.185	0.166	0.064	−0.131		0.170		0.176		0.266
Arg	4	0.833	0.947	0.852	0.153	4.768	0.408	−0.191	−0.317	−0.153	0.459		−0.001				0.298	−0.086	0.138
Gln	4	0.864	0.906	0.647	0.633	4.47	0.573	−0.265	−0.440	−0.282	0.088		−0.189	−0.017			0.127		0.137
Gly	4	0.859	0.839	0.570	0.267	5.721	0.284	−0.248	−0.330	0.057	0.133	0.348	0.083	−0.344	0.245			−0.148	
Pro	4	0.857	0.861	0.678	0.247	4.837	0.278	−0.160	−0.301		0.309	0.131	−0.048	0.010	0.096	0.106	0.061	−0.065	
Ala	4	0.965	0.782	0.506	0.372	5.761			−0.609	0.218		0.099	−0.143		0.319				
Asn	4	0.931	0.920	0.824	0.178	4.967	0.340	−0.260	−0.348		0.246		−0.175	−0.201					0.166
Cit	4	0.864	0.953	0.794	0.140	5.802	0.349		−0.227	0.333	−0.055	−0.084	0.126		0.240			0.301	−0.254
GAB	4	0.939	0.809	0.507	0.010	5.118	0.336		−0.371	−0.462	0.476		0.379	0.243					−0.559

[a] R^2x, R^2y: sum of squares of the independent (x) and dependent (y) variables explained by the PLS model; Q^2: total variation that can be predicted by the model; *RMSEcv*: root mean square error of cross validation.
[b] Full names of amino acids can be found in footnotes of Table 2.

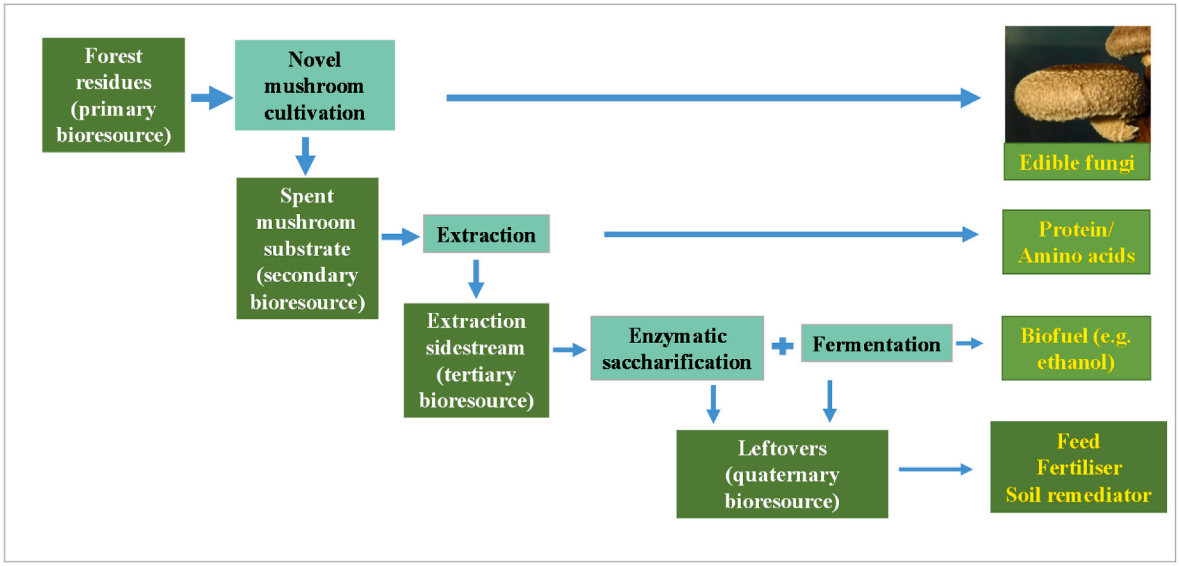

Fig. 5. Schematic of possible shiitake-based cascade process for food, fuel and other bio-based products. The illustration is modified from Fig. 3 of Martin et al. (2023).

indirectly use it as a metabolite, depending on the individual AA and/or biochemical reactions associated with the substrate properties and specific microenvironment near the fungal hyphae. Bianchi et al. [26] and Arsenault et al. [27] proposed that fungi could benefit energetically by taking up AAs available in substrates for direct use rather than synthesizing them. However, the addition of 2% whey in this study did not result in FB-EAA levels comparable to those in published literature (Table S1). Differences in substrate composition could explain this discrepancy. It was also possible that 2% whey addition was still an insufficient nitrogen supply, or that the efficiency of bioconversion/biotransformation of AA/nitrogen to the FBs was too low. A low efficiency of bioconversion/transformation of an AA molecule from substrate to FB may be due to physical (e.g., distance not reachable), chemical (e.g., inhibitory components released from the wood), or biological (e.g., low enzyme activity) factors.

The present study indicated that, as expected, substrate species had an influence on AA content. Differences in substrate species can lead to distinct compositional changes in SMS [8] following the fungal degradation of lignocellulosic substrates. This can influence not only mycelial growth, but also the AA composition in the substrate, and subsequently, the bioconversion/transformation of AAs into FBs. The relationship between substrate species and AAs exhibited both linear and non-linear trends, with either positive or negative effects, depending on the specific substrate species (and species combinations) and type of AAs (in SMS or FB). For instance, birch tended to positively affect TAA and EAA content in FBs, whereas alder had a negative effect. Aspen's impact was variable, depending on the specific AAs (Fig. 3B). In SMS, the highest TAA was associated with the highest alder fraction but the lowest birch fraction. These findings, derived from D-optimal designed experiments and associated PLS models (Figs. 2–3), underscore the importance of varying the substrate ingredients to adjust the AA profile and content of shiitake mushrooms toward a more desirable protein content and AA composition. Optimization of substrate formulations is also essential for effective SMS valorization. However, further exploration of the chemical factors behind the relationships between AA composition and the substrate species is needed.

By analyzing correlations between the major chemical components in IMSs and AAs in SMSs and FBs, this study takes a step further toward understanding the mechanisms behind the effects of different substrate ingredient species. The multivariate analysis through PLS regression allowed exploration of the components in the IMS that are important for each AA in FBs and SMS.

One of the major findings was that nitrogen-related variables entered the regression models more often than lignocellulosic ones. Total nitrogen (TN) was positively correlated to the AA contents in whichever model it was entered, whereas TC/TN, which influenced all models, was negatively correlated. This aligned with expectations since AAs are nitrogen carriers in fungal mycelia and FBs, similar to other organisms where AAs play important roles in various pathways involving nitrogenous metabolism [28]. Surprisingly, soluble NO_2^--N exhibited more influence on AAs than NH_4^+-N and NO_3^--N. Among the lignocellulosic variables, acid soluble lignin had the most impact, whereas Klason lignin had the least effect on AAs. These unexpected results open avenues for further investigation into the complex interactions between nitrogen sources and lignocellulosic components in shaping AA profiles.

Another key finding was that significant PLS models were achieved for 19 of the 22 analyzed AAs in both FB and SMS, using the experimental factors (Tables 2 and 3) or the 13 substrate chemical constituents (Table 5) serving as predictors. However, AAs Asp, Ser, and Tyr did not yield significance.

The models for the experimental factors (Tables 2 and 3) showed overlapping significant results, but when using only the substrate chemical constituents as independent variables (Table 5), SMS had 15 significant models whereas FBs had only 5. This suggests the AAs in FBs are less dependent on the initial substrate chemistry than AAs in SMS. It seems that the shiitake fungus has revolved an ability to utilize various chemical components for a stable AA composition in FBs. Conversely, the AA composition in SMS reflects greater adaptability of the mushroom mycelia to the substrate.

Interestingly, the PLS modeling did not overlap for the FBs and SMSs except GAB (Table 5). His, Lys, Glu, and Orn, which primarily existed in the free form, showed significant regressions with the substrate constituents in FBs, suggesting that these free AAs are crucial for shiitake FB development.

The roles and significance of AAs in FBs and SMSs are contingent upon the metabolic processes during the shiitake fungus growth. However, comprehensive interpretation of the mechanisms underlying these effects is limited by the current data constraints. This highlights the need for further metabolite system analysis in future studies.

4.3. Potential co-production of protein and biofuels from SMS

The findings that the sum of 22 AAs accounted for up to 51 mg per gram DM, i.e., 5.1% of SMS, suggesting that SMS could be a valuable feedstock for extracting protein and AAs. Extracting AAs and proteins from SMS before converting the remnants into biofuels may enhance SMS valorization and improve resource efficiency.

One of the key findings of this study was that the EAA profile in FBs lay within the desired protein quality range, even when grown on initial substrates without whey addition. Their total nitrogen (TN) contents ranged from 0.6 to 0.7% DM, depending on the substrate species (Table S2), which aligns with levels associated with enhanced delignification of the substrate by shiitake reported in a previous study [7]. SMS, i.e., partially delignified substrate, is rich in cellulose, making it a better feedstock than raw lignocellulosic residues for the production of cellulose-based ethanol. These findings support an integrated approach for producing both mushroom protein and, e.g., bioethanol fuels. They also highlight the importance of developing technologies for efficiently extracting protein and then producing ethanol biofuel from SMS, which should be included in future studies.

5. Conclusion

The fruit bodies (FBs) of shiitake exhibit a high-quality AA profile, with total essential amino acids (EAA) ranging between 248 and 313 mg per g protein, despite cultivation on low N-content lignocellulosic substrates. By adding up to 2% N-rich whey to initial substrate (IMS), the sum of EAAs increased to 263-394 mg per g protein. In total, the AA content varied between 8.9 and 12.9% of DM in FBs and 2.7-5.1% in spent mushroom substrates (SMS). Thus, SMS represents a protein source alongside FBs, in addition to being a preprocessed feedstock for producing second-generation biofuels, i.e., bioethanol. Among the 13 independent IMS variables analyzed using multivariate modeling, the C/N ratio and soluble NO_2-N exerted the strongest influence on the AA content. The C/N exerted a negative effect, whereas soluble NO_2-N influenced the AAs in FBs positively but negatively in SMS. Klason lignin had the least influence. Interestingly, acid soluble lignin also had a positive influence on AAs in FBs. These findings could help to optimize substrate formulations to achieve desired mushroom food-based production and valuable byproducts using lignocellulosic residual materials. In addition, the results suggest potential for further exploration, e.g., extraction of proteins and AAs in SMS prior to processing the remaining wood remnants into bioethanol.

CRediT authorship contribution statement

Feng Chen: Writing – original draft, Methodology, Investigation, Data curation. **Torbjörn A. Lestander:** Writing – review & editing, Validation, Data curation. **Jonas Burén:** Writing – review & editing, Writing – original draft, Data curation. **Anna Sjödin:** Writing – review & editing, Data curation. **Calle Niemi:** Writing – review & editing, Validation. **Michael Finell:** Writing – review & editing. **Shaojun Xiong:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics statement

This study is not applicable to ethical issues.

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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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2026.102799.

Abbreviations

AA, amino acid; TAA, total amino acid; EAA, essential amino acid; FB, shiitake fruit body; IMS, initial mushroom substrate; SMS, spent mushroom substrate; DM, oven-dry mass; DW, dry weight; C/N, ratio of carbon to nitrogen; TN, content of total nitrogen; TC, content of total carbon; PCA, principle component analysis; PLS, partial least squares regression; His, histidine;, Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Trp, tryptophan; Val, valine; Arg, arginine; Gln, glutamine; Gly, glycine; Pro, proline; Tyr, tyrosine; Ala, alanine; Asn, asparagine; Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Cit, citrulline; GAB, gamma-aminobutyric acid; Orn, ornithine.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. A part of data (contents of cellulose and hemicellulose in initial substrates) presented in Supplementary Table S2 are from previous publication and can be found at https://doi.org/10.1016/j.biortech.2021.126256. All data are peer reviewed before the publication. Readers can find all data at this journal website as a data repository.

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