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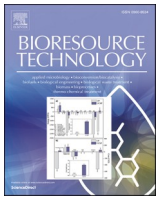

Upcycling of melanoidin-rich Chinese distilled spent grain through solid-state fermentation by *Aspergillus awamori*

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HIGHLIGHTS

- Chinese distilled spent grain (DSG) is a good substrate for growing *A. awamori.*
- SSF facilitated the solubilization of proteins and phenolic compounds from DSG.
- *A. awamori* effectively degraded DSG melanoidins during SSF.
- DSG melanoidins can be converted into *A. awamori* and *A. oryzae* biomass.

GRAPHICAL ABSTRACT

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Chinese liquor
Solid waste
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ABSTRACT

This study investigated the upcycling of distilled spent grain (DSG), a melanoidin-rich by-product of the Chinese liquor industry, via fungal solid-state fermentation (SSF). Two fungi, *Aspergillus oryzae* and *Aspergillus awamori*, were tested, with *A. awamori* growing better on DSG than *A. oryzae*. SSF with *A. awamori* increased the concentration of water-soluble protein and phenolic compounds in DSG extracts by 46.5 % and 52.5 %, respectively, and reduced melanoidin level by 73.5 % w/w of DSG, suggesting *A. awamori* could metabolize melanoidins. Submerged fermentation (SmF) using isolated DSG melanoidins as sole carbon and nitrogen sources confirmed this observation. After 3 days of fermentation, *A. awamori* and *A. oryzae* biomass reached 2.5 g/L and 1.5 g/L, quenching melanoidin color by 24.4 % and 12.4 %, respectively. SmF by *A. awamori* also released free arabinose, glucose, and xylose. Data highlighted the possibility of converting melanoidins into edible mycelia resources, potentially applicable to various melanoidin-rich food by-products.

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1. Introduction

Chinese liquor (*Báijiǔ*) is a traditional fermented beverage made from grains such as sorghum, wheat, rice, and corn. After several months of spontaneous fermentation, the fermented grains are mixed with rice husks and subsequently distilled to produce liquor. The remaining solids after distillation are called distilled spent grain (DSG). DSG is the primary by-product of *Baijiu* production with an average annual output over 30 million tons (Ma et al., 2023). Fresh DSG is susceptible to microbial spoilage, causing unpleasant smells and harmful organism growth, which negatively impact the environment. Currently, DSG is mainly used as low-cost poultry feed and fertilizer (Liu et al, 2022), missing the opportunity to use its potentially valuable components.

DSG is rich in insoluble dietary fibers (17–29% w/w). It also contains starch (10–15% w/w), protein (17–28% w/w), and fat (5–11% w/w), as well as bioactive compounds including organic acids, polyphenols, polypeptides, free amino acids, and melanoidins. Bioactive compounds from DSG used as functional foods or food additives presents a promising upcycling strategy (Liu et al, 2022). Their solubility enhances absorption and usability. However, extracting protein- and phenolic-rich soluble fractions is challenging due to their strong physical and chemical interactions with cellulose and hemicellulose (Deng et al., 2023). Enzymes like cellulases, hemicelluloses, and pectinases have been used to break down these polymers to release proteins and phenolic compounds. Fermentation can also be used for this purpose. Solid-state fermentation (SSF) is an effective and eco- friendly technique with lower operating costs and water consumption compared to submerged fermentation. This process can release and synthesize bioactive compounds through enzymatic hydrolysis and microbial metabolism (Thomas et al., 2013; Vilas-Franquesa et al., 2024).

Filamentous fungi are frequently used in SSF due to their excellent growth performance in low-water environments (Dulf et al., 2023). Being saprophytic organisms, they secrete powerful hydrolytic enzymes to release the nutrients needed for their growth (Conesa et al., 2001). *Aspergillus oryzae* and *Aspergillus awamori* are two edible and commercially available filamentous fungi that have been used in the production of various fermented foods such as soy sauce, miso, and sake. They can efficiently degrade complex components like cellulose, hemicellulose, starch, and proteins by releasing corresponding enzymes (de Castro et al., 2015), which make them useful in SSF of agro-food waste like DSG. Their edible mycelia (fungal biomass) also stand out as a sustainable alternative protein source, potentially applicable in food production (Strong et al., 2022).

Melanoidins are a large part of DSG that could be released through enzymatic action by fungi. They are mainly formed in high-temperature distillation, with a small amount formed during fermentation in *Baijiu* production (Fig. 1) (Yang et al., 2023). As the final product of the Maillard reaction, melanoidins are widely found in many thermally treated, fermented foods, and their byproducts. They significantly contribute to the food color and flavor, and provide various health-related benefits (Morales et al., 2012). However, their dark color is often undesired therefore physical technologies including absorption and filtration have been applied to decolorize or remove food melanoidins (Carrín et al., 2007). These methods diminish the flavor and aroma of foods and are not cost-effective for large-scale treatment (Miyagi et al., 2013). Although the formation of food melanoidins has not been elucidated yet, previous research focused on DSG melanoidins synthesis showed that proteins and phenolic compounds content increased in melanoidins, while carbohydrate content decreased during Chinese liquor fermentation, potentially indicating the microbial impact (Yang et al., 2023). The presence of carbohydrates and proteins in various food melanoidins suggests that fermentation may be an alternative strategy for melanoidin solubilization/removal (Tagliazucchi and Verzelloni, 2014), since microorganisms could use melanoidins as feed to in turn achieve melanoidin degradation. There are currently no reports investigating the degradation of food melanoidins or the effect of SSF on DSG by food-grade fungi.

This study aimed to explore the potential of fungal SSF to upcycle the melanoidin-rich DSG. We hypothesized that fungal SSF could increase the solubility of proteins and phenolic compounds while also solubilizing and/or degrading complex melanoidins in DSG. Hence, the level of soluble proteins, phenolic compounds and melanoidins were measured in SSF. Submerged fermentation (SmF) using DSG melanoidins as the sole carbon source and nitrogen source was conducted to investigate the fungal ability to degrade melanoidins. Our study demonstrates that food-grade fungi can be used to degrade food melanoidins both in solid and liquid-state fermentation processes. The use of edible fungi, *A. awamori* and *A. oryzae,* provides a safe, sustainable approach to upcycle DSG for food applications.

2. Materials and methods

2.1. Materials

DSG was kindly provided by Golden Seed Distillery Co., Ltd (Fuyang, China). The fresh DSG was dried in the oven at 70 ℃ overnight. The dried DSG with a moisture content of 12.2% was then sealed and stored in a cool, dry place. The chemical composition of dried DSG is 27.8%

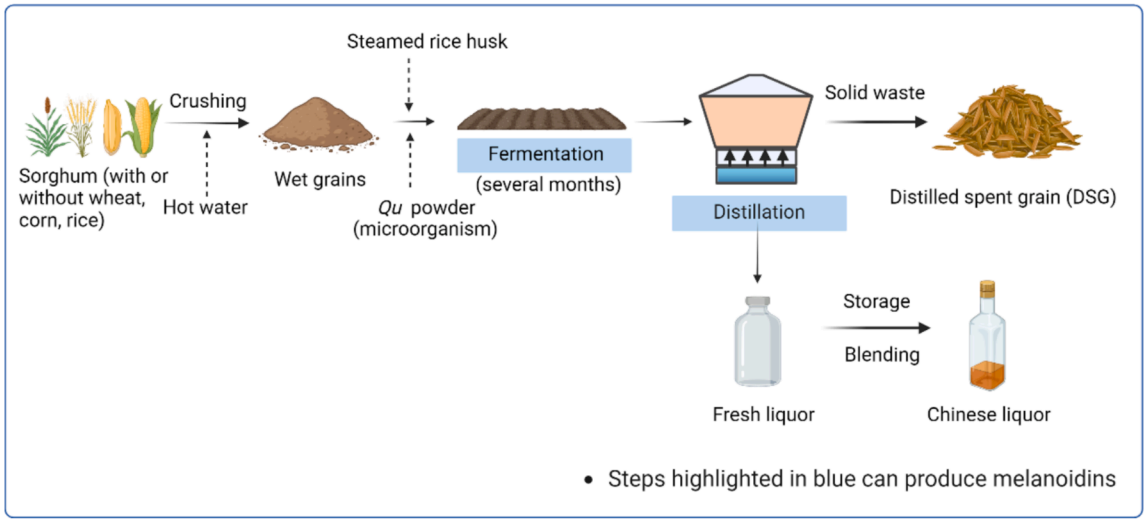

Fig. 1. The main process of Chinese liquor brewing (*Báijiǔ*).

available carbohydrate, 56.4% total dietary fiber, 12.2% crude protein, 2.7% total fat, and 0.9% ash.

A. oryzae was obtained from StarterCulturesEU (Amsterdam, The Netherlands) and isolated from powdered *koji* starter culture (Barley *koji* starter culture). *A. awamori* CSB 139.52 from Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) was kindly supplied by the Food Microbiology group of Wageningen University. Both fungal cultures were activated before SSF on Potato Dextrose Agar (Merck, Darmstadt, Germany) (see Supplementary Materials).

2.2. Solid-state fermentation of DSG

DSG was first ground into a powder using a 6875D Freezer/Mill® (Spex Sample Prep, United States). For SSF, 5 g of DSG powder was placed in a Petri dish and 1 mL of spore suspension (10^6 spores/mL) was added. The water content of the matrix was then adjusted to 64% by adding sterilized demineralized water. Instead of spore suspension, 1 mL of sterilized demineralized water was added to DSG serving as the control. The DSG mixture was incubated at 30 °C for 5 days, and samples were collected on days 0, 2, 3, 4, and 5. The fermentation experiment was performed in duplicate, and all analyses were run in triplicate. The collected samples were stored at –20 °C for further analysis.

2.3. Aqueous and alkaline extraction of DSG

The aqueous and alkaline extracts were obtained from the raw, control, and fermented DSG using the methods of Yang et al. (2023). To prepare the aqueous extracts, Milli-Q water was added to DSG samples at a ratio of 20:1 (v/w) and then stirred at 350 rpm for 1h at room temperature. The supernatant obtained from centrifugation was filtered by 0.45 μm RC filter to remove hyphae and spores. The pellet remaining was extracted twice with 0.11 M NaOH to prepare the alkaline extracts. The volume of NaOH added was the same as water used in the aqueous extraction. A single extraction was conducted at 50 °C for 1h with stirring at 350 rpm. After centrifugation, supernatants from two alkaline extractions were combined and the pH was adjusted to 4.6 using 2N HCl to precipitate the proteins. The precipitated proteins were removed by centrifugation and the resulting alkaline supernatant was collected and stored at –20 °C for further analysis. All centrifugation mentioned was performed at 4,000 × *g* for 20 min.

2.4. Browning, pH, dry matter, and color of the aqueous and alkaline extracts

The browning of the aqueous and alkaline extracts was measured at the absorbance of 420 nm by a microplate reader (Molecular Devices, LLC, California, USA). pH was determined with a calibrated pH meter (VWR International, Boxmeer, Netherlands). Dry matter was measured according to the protocol AOAC 950.46 (AOAC, 2005). Color determination was carried out using a color meter (ColorFlex, HunterLab, Reston, VA, USA) (illuminant D 65) with a cylindrical glass cuvette of 3.3 cm inner diameter and 2.2 cm inner height. A white plate was used to standardize the instrument. The color was expressed by L* (lightness), a* (redness, + or greenness, −), and b* (yellowness, + or blueness, −) (Echavarría Vélez et al., 2013). The color difference was calculated by ΔE* according to Equation $\Delta E^* = ([(\Delta a^*)^2+(\Delta b^*)^2+(\Delta L^*)^2]^{1/2})$.

2.5. Melanoidins isolation and quantification

The melanoidins in aqueous and alkaline extracts were isolated by ultrafiltration using centrifugal tubes (Sartorius Stedim Lab Ltd., Gloucestershire, UK) with molecular weight (MW) cut-offs of 10 kDa and 100 kDa. After multiple rounds of centrifugation, two melanoidin fractions, 10–100 kDa and >100 kDa, were collected and freeze-dried. The content of melanoidins was estimated by weight difference.

2.6. Protein, free amino acids, sugar, total phenolic content, and antioxidant activity of the aqueous extracts

The protein content in the aqueous extracts was analyzed using Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), expressed as mg bovine serum albumin equivalents per 100 mg of dry DSG (mg BSA/100 mg). The free amino acids (FAAs) were measured by OPA method (Mulet-Cabero, et al., 2017). The results were expressed as mg serine equivalents per g of DSG on a dry basis (mg/g). The sugar analysis was carried out by High-Performance Anion Exchange Chromatography (HPAEC, Dionex ICS-5000-Ion Chromatography System, Thermo Fischer Scientific) with a CarboPac PA-1 column (2 × 250 mm), using the method described in Gilbert-López et al. (2015) with a minor modification; the results were expressed as mg per 100 g of dry DSG (mg /100 g). The total phenolic content (TPC) was determined using Folin–Ciocalteau procedures (Singleton et al., 1999), expressed as mg gallic acid equivalents per 100 mg of dry DSG (mg GAE/100 mg). The antioxidant activity was assessed by the DPPH (2,2-di-phenyl-2-picryl-hydrazyl) method, reported as mmol Trolox equivalents per g of dry DSG (mmol TE/g) (see Supplementary Materials).

2.7. Enzyme assays

Amylase activity was assayed by α-amylase assay kit (Ceralpha Method) from Megazyme (K-CERA, Neogen Europe Ltd., Ayr, United Kingdom). One Unit of activity is defined as the amount of enzyme required to release one micromole of *p* −nitrophenol in one minute in the presence of excess thermostable α-glucosidase. α-amylase activity was expressed as CU/g dry DSG. Protease activity was measured as described by Sandhya et al. (2005). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μM tyrosine per min under the assay conditions. The results were expressed as U/g dry DSG (see Supplementary Materials).

2.8. Submerged fermentation (SmF) of melanoidins from alkaline extracts of raw DSG

Melanoidins with MW over 100 kDa from alkaline extracts of raw DSG were resuspended in sterilized water at 5 mg/mL and inoculated with *A. awamori* and *A. oryzae* (10^6 spores/mL). Melanoidin solution without adding fungi served as control. All media were incubated at 30 °C for 5 days with orbital shaking at 200 rpm by rotator (Biosan SIA, Riga, Latvia). Samples were collected at 0, 1, 2, 3, 4, and 5 days. The SmF was conducted in duplicate, and all relevant analyses were performed in triplicate.

2.9. Decolorization, monosaccharide, and protein analysis of SmF resulting materials

The collected melanoidin media of SmF process were filtered through filter paper (Whatman, No. 44) and centrifuged at 4,000 × *g* for 10 min. Decolorization was assessed by measuring the browning reduction at 420 nm using a microplate reader. Protein content was determined as described in section 2.6, expressed in mg BSA/mL. Monosaccharide analysis followed the method outlined in section 2.6.

2.10. Quantification of fungal biomass growth during SSF

In SSF, fungal biomass was estimated based on the ergosterol content, using the method of Nayan et al. (2018) with some modifications. Fermented DSG was dried in a conventional oven to constant weight at 60 ℃, and homogenized. Saponification was achieved by mixing 200 mg of the dried sample with 2 mL of KOH/methanol solution (1:9), shaking the mixture at 200 rpm for 30 min at 80 °C. After cooling to room temperature, 1 mL of distilled water and 2 mL of hexane were added to

the mixture and shaken vigorously. The hexane layer (upper layer) was then collected, and the extraction process was repeated three times.

The enriched hexane layer containing ergosterol was dried by a sample concentrator (Stuart SBHCONC/1, Cole-Parmer Instrument Company, Illinois, USA) and then re-dissolved in 2 mL methanol (≥99.8%). Cholesterol was prepared in methanol (stock solution, 5 mg/mL) and added to samples as an internal standard with 0.1 mg/mL (final concentration). The solution was filtered by 0.2 μm PFTE membrane before analysis. The measurement of ergosterol content was performed on a reversed-phase prevail organic acid column (250 x 4.6 mm, Phenomex aqua 5 μm) by UHPLC-PDA analysis (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The mobile phase was 90% methanol (A) and 10% (1:1) 2-propanol/hexane (B). The flow rate was 1 mL/min. The ergosterol and cholesterol peaks were detected at 280 nm and 210 nm, respectively. The calibration curve was established by plotting the peak area ratio of ergosterol to cholesterol against their concentration ratio. The concentration used in the calibration curve for ergosterol was 0.05–0.8 mg/mL, and for cholesterol was 0.05 mg/mL. R^2 of the calibration curve was 0.997. Pure fungi grown on potato dextrose agar (PDA) medium was collected with different weight. The correlation between ergosterol content and pure fungal biomass (weight) was established to estimate the fungal biomass in fermented samples.

2.11. Quantification of fungal biomass in SmF

In SmF, fungal biomass was represented by the mycelia weight. The fermented melanoidin medium was filtered by filter paper (Whatman, No. 44) and the fungal pellets were washed with demineralized water. The obtained mycelia were weighed after drying at 80 °C until no further weight change was recorded.

2.12. Statistical analysis

Statistical analysis was performed using SPSS software version 26.0 (IBM Inc., New York, USA). Data were expressed as mean ± standard deviation (SD). Significant differences among the means were determined by one-way analysis of the variance (ANOVA) after testing for normal distribution (Shapiro–Wilk test) and homogeneous variance (Levene's test). The post hoc test with Tukey's Honestly Significant Difference (HSD) test was then performed. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussions

3.1. Characterization of SSF on DSG by A. awamori and A. oryzae

A. awamori and *A. oryzae* were cultured on DSG for 5 days. *A. awamori* successfully colonized the DSG with visible growth of mycelium and spores, whereas no noticeable growth was shown by *A. oryzae* (see Supplementary Materials). During the first three days of *A. awamori* fermentation, significant mycelial growth was observed, with sporulation starting after day 3, and visible accumulation of spores by day 5.

As shown in Fig. 2a, the aqueous extracts of DSG underwent a substantial color change during SSF by *A. awamori*, the browning intensity increased almost 2.5-fold after 5 days (see Fig. 2b). Notably, no color change of DSG aqueous extracts fermented by *A. oryzae* were shown. In Fig. 2c, the pH values during SSF were shown: fermentation by *A. awamori* raised the pH from 3.70 to 5.24, implying ammonia or amino acid production (Nursiwi et al., 2019). Similarly, the pH values of the aqueous extracts after fermentation by *A. oryzae* were very close to those of the unfermented samples. SSF by *A. awamori* reduced the water-soluble content of DSG, decreasing dry matter from 15.3 g/100 g DSG to 10.2 g/100 g DSG (Fig. 2d). This effect could be attributed to the consumption of soluble substances such as free sugars initially present in the DSG. He et al. (2024) also reported a decrease in water-soluble substances following SSF.

The results demonstrated that *A. awamori* exhibited superior growth and a greater ability to use DSG as substrate than *A. oryzae*, probably due to differences in their metabolic responses and enzymatic capabilities

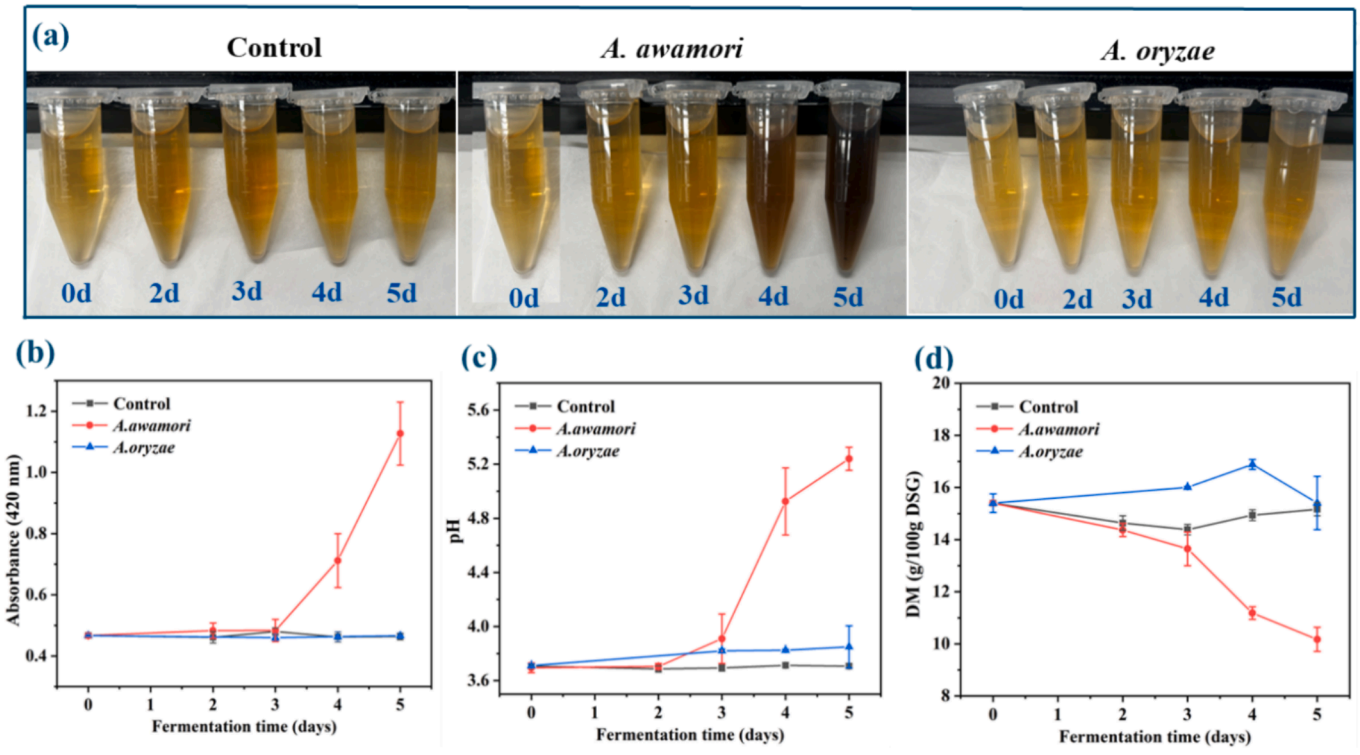

Fig. 2. Panel (a): aqueous extracts obtained after fermenting DSG by *A. awamori* and *A. oryzae* over different time periods; Panel (b): Browning index (absorbance value at 420 nm); Panel (c), pH values; Panel (d), dry matter values.

when faced with complex substrates. Manan and Webb (2016) using three different solid wastes (wheat bran, soybean hulls, and rapeseed meal) as substrate found *A. awamori* was a good producer of cell wall degrading enzymes such as cellulase and xylanase, while *A. oryzae* was more efficient in producing proteases. The enzymatic profile of *A. awamori* may confer an advantage in utilizing cellulose and hemicellulose, which are the main fiber components in DSG. DSG also contain approximately 13% of starch (Ma et al., 2023) and while enzymes from *A. awamori* can degrade nearly all the starch in wheat flour, those from *A. oryzae* can only degrade a small portion of it (Wang et al. 2009). *A. oryzae* may require specific conditions or additional substrates to grow effectively on DSG. In this vein, Wang et al. (2009) found that combining enzyme solutions from *A. oryzae* and *A. awamori* can significantly increase the hydrolysis degree of both starch and protein.

Considering our results and the findings of literature, we decided to conduct in depth SSF experiments on DSG, using only *A. awamori*.

3.2. Growth of A. awamori on DSG

The measurement of ergosterol was conducted to estimate the growth of *A. awamori* on DSG. As depicted in Fig. 3a, the ergosterol content in DSG increased slightly after 2 days of SSF, with no statistical difference compared to day 0. On the third day of SSF, ergosterol accumulated considerably, reaching 13.2 mg/g. Following 3 days, there was a decrease in ergosterol content, likely due to the metabolic shifts of fungi by reallocating resources to sporulation and metabolite production (Wang et al., 2019). As sporulation progressed, ergosterol production increased again to 13.9 mg/g on day 5. This rise may be explained by the fungus stabilizing its metabolism to produce viable fungal biomass in a high spore population. A small amount of ergosterol was detected on day 0, which may be due to the residual fungal cells in DSG from *Baijiu* fermentation.

Ergosterol is known to be a membrane-associated sterol found nearly exclusively in fungi. The amount of ergosterol in fungi varies greatly depending on several factors, including the growth environment and stage of fungus, fungal species, and substrate composition. Gutarowska and Żakowska (2010) and Stoffel et al. (2019) all conducted SSF using wheat as the solid substrate. The ergosterol content was 0.04 mg/g, 0.2 mg/g, and 0.6 mg/g with *Aspergillus ochraceus*, *Agaricus blazei*, and *Pleurotus albidus*, respectively. The presence of lignin in the substrate can also promote mycelial growth and ergosterol accumulation (Wu et al., 2023). Ergosterol data showed DSG was an excellent substrate for *A. awamori* with vigorous growth.

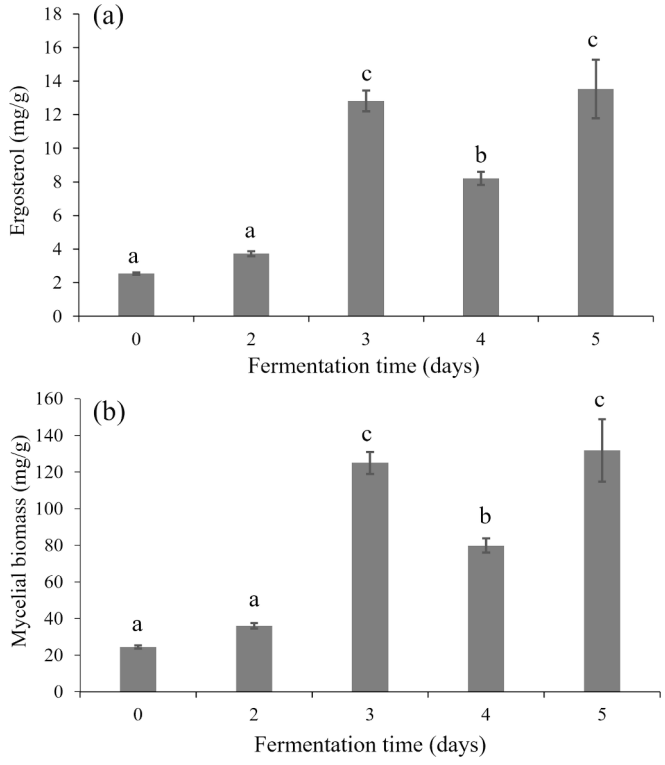

Fig. 3. Ergosterol content (a) and mycelial biomass content (b) during SSF of DSG by *A. awamori*. Different letters represented significant differences ($p < 0.05$).

Fig. 3b displays the *A. awamori* biomass in fermented DSG. There is a good consistency between *A. awamori* biomass and ergosterol content during SSF of DSG. The higher and statistically similar level ($p < 0.05$) of fungal biomass was determined on day 3 and 5 to be 124.9 mg/g and 131.8 mg/g, respectively. This finding suggests that 3 days could be an optional time point for harvesting mycelial biomass. To our knowledge, the biomass of *A. awamori* under solid-state fermentation is currently not well-documented. Comparable biomass levels of *A. oryzae* and *Rhizopus oligosporus*, ranging from 56 to 132 mg/g, were recorded in the study of Zwinkels et al. (2023) using barley and rice as substrates. In general, it is expected that the fungus mycelium might account for 10–15% of the dry matter at the harvesting time.

3.3. Color analysis of A. awamori SSF extracts

The most noticeable characteristic of DSG after SSF by *A. awamori* is the browning of aqueous extracts (Fig. 2a), indicating the production and/or release of colored compounds. The color of alkaline extracts of DSG was darker than that of water extract at time zero (see Supplementary Materials), which was consistent with the report from Akogou et al. (2018). Following fermentation, the color of water extracts was more intense than the alkaline extracts, indicating that more water-soluble colored compounds may be formed via SSF by *A. awamori*.

Table 1 presented the dry matter and color parameters of aqueous and alkaline extracts of DSG. Alkaline extracts contained 2.2-fold more soluble materials than aqueous extracts before fermentation, and 3.3-fold more after 5 days of SSF. Fermentation reduced dry matter in aqueous extracts but not in alkaline extracts ($p<0.05$). Interestingly, the browning of aqueous extracts increased from 0.1 to 0.37 after SSF by *A. awamori*, whereas the browning of alkaline extracts decreased from 0.43 to 0.2.

The change in melanoidin concentration could partially account for the variations in dry matter and browning during SSF. Melanoidins have reduced concentrations in both aqueous and alkaline extracts of DSG following SSF (see section 3.5). The reduction in water-soluble melanoidin may play a role in the decrease of dry matter of aqueous extracts while the degradation of alkaline-soluble melanoidins could partly account for the reduced browning of alkaline extracts. The production of non-brown substances during SSF compensated for the dry matter loss in alkaline extracts. The metabolism of alkaline-soluble melanoidins by *A. awamori* could release low MW water-soluble substance (see section 3.5), thus contributing to the browning of DSG aqueous extracts.

After fermentation, the aqueous extracts had a lower L* value, indicating it became darker. The red-yellow color in aqueous extracts was increased. The alkaline extracts were green and yellow before SSF, as shown by lower a* and higher b* values. It tended to be reddish and bluish after SSF. SSF altered the color profile of DSG extracts.

The overall color changes in the DSG extracts after SSF could be due to the following three reasons: first, bio-pigments can be produced by the fungal metabolism during fermentation. Erythroglaucin (red), catenarin (red), asperenone (yellow), and viomellein (reddish-brown) were generated by various *Aspergillus* species (Lagashetti et al., 2019). Melanin-like pigments, such as pyomelanin (brown-black), are often associated with fungal spore production and may serve protective functions for the spores (Schmaler-Ripcke et al., 2009). These pigments could be secreted into fermented substrates, leading to an increase in darkness. An example of *A. niger* showed that black pigment production

Table 1
The dry matter, browning, and chromaticity of aqueous and alkaline extracts from DSG with SSF by *A. awamori*.[A]

Fermentation period (day)	Extraction way	Dry matter (g/100 g DSG)	Browning[B]	Color[B]			
				L*	a*	b*	ΔE*
0	Water extraction	15.39 ± 0.35 [b]	0.10 ± 0.01 [d]	3.69 ± 0.18	−0.58 ± 0.10	−0.29 ± 0.06	3.75 ± 0.19
0	Alkaline extraction	33.29 ± 1.68 [a]	0.43 ± 0.01 [a]	3.88 ± 0.09	−0.71 ± 0.06	−0.15 ± 0.01	3.94 ± 0.08
5	Water extraction	10.17 ± 0.46 [c]	0.37 ± 0.01 [b]	3.63 ± 0.07	−0.45 ± 0.08	−0.16 ± 0.02	3.67 ± 0.07
5	Alkaline extraction	33.74 ± 2.07 [a]	0.20 ± 0.01[c]	4.30 ± 0.17	−0.56 ± 0.04	−0.63 ± 0.53	4.41 ± 0.24

[A] The values were presented as mean ± standard deviation (SD) (n=3). Values in the same column with different letters mean significant differences ($p < 0.05$).
[B] The measurement of browning and color was carried out to extracts at 1mg/ml.

is linked to conidia germination and may be regulated by serine-threonine protein kinases (Shankar, 2022), with similar mechanisms likely present in *A. awamori*. Second, the transformation of phenolic compounds by the catalysis of enzymes. Polyphenol oxidases like tyrosinase, catechol oxidase, and laccase can produce highly reactive quinones that polymerize to generate brown colorants (de Carvalho et al., 2024). Third, chromophores such as pyrroles, imidazoles, and their nitrogen-containing derivatives are present in melanoidins (Wang et al., 2011). The release of coloring compounds from melanoidins could affect the color of DSG extracts.

3.4. Degradation products of DSG by A. awamori

3.4.1. Soluble proteins, phenolics, and antioxidant activity of DSG extracts

Data on protein and phenolic contents in the aqueous extracts of DSG were reported in Table 2. There was an increased trend of protein and phenolic levels as fermentation advanced. Initially, the soluble protein and phenolic compounds content were 29.3 mg/100 mg and 3.3 mg/100 mg, respectively. From day 4 of fermentation, the protein and phenolic levels were significantly higher than the control ($p < 0.05$). After 5 days, the protein and phenolic content were increased to 54.6 mg/100 mg and 5.1 mg/100 mg with an improvement percentage of 46.5% and 52.5%, respectively. The antioxidant activity of aqueous extracts rose considerably by 1.12 times after SSF. SSF facilitated the solubilization of proteins, phenolic compounds, and antioxidants in DSG.

Table 2
Protein, total phenolic content, and antioxidant activity of aqueous extracts from control and fermented DSG by *A. awamori*.

Samples	Fermentation period (day)	Protein content (mg BSA/100 mg)	Total phenolic content (mg GAE/100 mg)	Antioxidant activity (mmol TE/g)
Control	0	25.16 ± 2.34 [a]	3.35 ± 0.06 [a]	0.07 ± 0.02 [a]
	2	29.59 ± 1.37 [a]	3.54 ± 0.02 [a]	0.05 ± 0.02 [a]
	3	28.84 ± 0.90 [a]	3.45 ± 0.07 [a]	0.09 ± 0.01 [a]
	4	29.71 ± 1.18 [a]	3.42 ± 0.05 [a]	0.06 ± 0.02 [a]
	5	28.93 ± 1.00 [a]	3.61 ± 0.04 [a]	0.06 ± 0.01 [a]
Fermented DSG	0	29.25 ± 0.81 [a]	3.33 ± 0.23 [a]	0.06 ± 0.02[a]
	2	30.10 ± 3.93 [a]	3.35 ± 0.10 [a]	0.06 ± 0.01 [a]
	3	32.89 ± 1.82 [a]	3.43 ± 0.11 [a]	0.07 ± 0.02 [a]
	4	44.16 ± 0.10 [b]	4.33 ± 0.19 [b]	0.12 ± 0.01 [b]
	5	54.63 ± 3.95 [c]	5.07 ± 0.06 [c]	0.14 ± 0.01 [c]

The results were shown as mean ± standard deviation (SD) (n=3). Values in the same column with different superscript letters were significantly different ($p < 0.05$).

Reports on the rise in protein content of DSG extracts mostly focus on crude protein. There are no explicit studies about the enrichment of water-soluble proteins in DSG by SSF yet. Comparable solubilization effects have been noted by SSF of brewery spent grain with *Rhizopus oligosporus* (Canedo et al., 2016). One possible explanation for the increase in soluble proteins following SSF is related to the enzymatic degradation of the complex lignocellulosic structures leading to the conversion of insoluble proteins into more soluble, smaller proteins and peptides, thereby increasing the content of water-soluble proteins (de Castro et al., 2015; Li and Wang, 2021). Ramírez et al. (2021) mentioned that fungal biomass production can also contribute to the increase in water-soluble proteins upon fermentation. The solubilization of phenolic compounds can be attributed to the enzymatic activity of *A. awamori*, such as glycosidases and esterase that hydrolyze glycosidic and ester bonds, releasing phenolic compounds that were previously insoluble (Saharan et al., 2020).

3.4.2. Free amino acids and sugar of DSG extracts

The content of FAAs and sugar in aqueous extracts of DSG are shown on Fig. 4. FAA levels increased significantly in the first 3 days of SSF ($p < 0.05$), peaking at 12.3 mg/g DSG on day 3 – a 1.6-fold increase

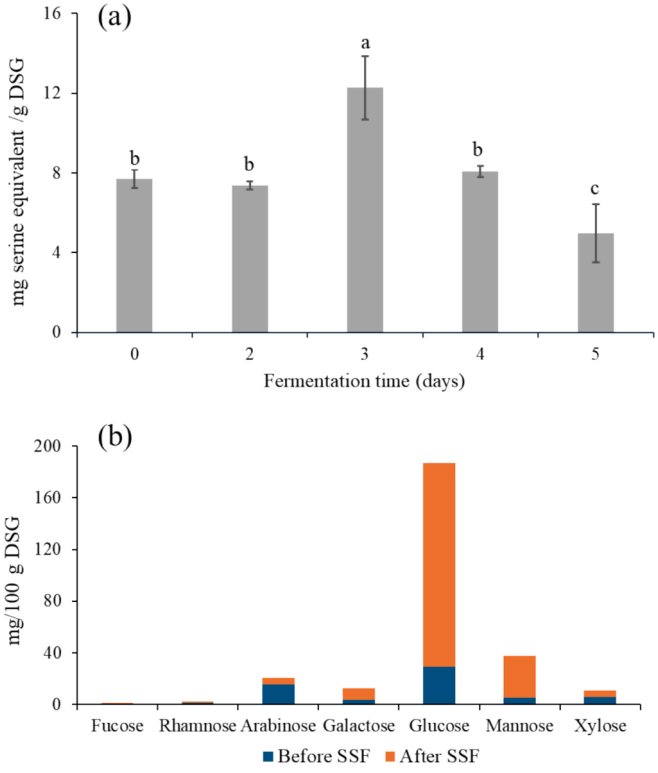

Fig. 4. The fermentation products in aqueous extracts of DSG by *A. awamori*. (a) the content of free amino acids at different durations of SSF; (b) the content of free sugars before and after 5 days of SSF.

compared to day 0 (Fig. 4a). Subsequently, a continuous decrease in FAAs content was observed. After 5 days of SSF, the content of FAAs was reduced to 4.9 mg/g DSG, lower than day 0. As for sugars, seven monosaccharides were detected in the aqueous extract of DSG before and after SSF (Fig. 4b). The concentrations of galactose, glucose, and mannose notably increased post-SSF, while rhamnose and xylose remained stable. Particularly, the glucose level rose greatly, with a 5.4–fold enhancement compared to pre-SSF concentrations.

The alterations in FAAs and monosaccharide content are likely driven by fungal metabolism (Tang et al., 2021). The initial increase in FAAs may result from the degradation of small-molecule peptides and protein by extracellular proteases produced by *A. awamori*. After 3 days, *A. awamori* appears to begin utilizing FAAs as a primary nitrogen source to support metabolic processes such as sporulation and new compounds synthesis. This hypothesis is further supported by the observed increase, rather than decrease, in soluble protein levels (Table 2), suggesting that FAA uptake is prioritized over protein degradation at this stage. Consequently, despite the continued rise in protease activity (see Supplementary Materials), the increased uptake of FAAs by *A. awamori* leads to a net reduction in FAA level in the aqueous extract. The rise in free sugars is similarly attributed to enzymatic hydrolysis, as *A. awamori* produces many enzymes that degrade cellulose, hemicellulose, and residual starch into simpler sugars, mainly glucose. When the sugar released exceeds its consumption by *A. awamori*, free sugars can accumulate in the substrate. Ichikawa et al. (2022) reported that the content of free sugars in fermented okra increased 4-fold after SSF by *Aspergillus* spp. compared to the unfermented sample.

The specific activities of protease and amylases, two crucial enzymes produced by *A. awamori* during SSF of DSG, are presented (see Supplementary Materials). Protease activity increased throughout SSF, reaching 159.3 U/g DSG after fermentation. Similar results were found in the SSF of wheat bran by *A. niger* (de Castro et al., 2014). Protease produced by *A. awamori* facilitated FAA release at the initial stage as a nitrogen source to support fungal growth and metabolism. Amylase activity also showed an increasing trend during SSF, notably surpassing the unfermented sample after 3 days. Amylase activity reached 13.8 CU/g DSG and 20.2 CU/g DSG on days 4 and 5, respectively. Amylase plays a role in polysaccharides degradation into simple sugar, serving as a carbon source for fungal fermentation. Depending on the substrate and the fermentation conditions, the amylase activity varied considerably. In the SSF of tomato pomace by *A. awamori*, the amylase activity was observed to be 21.5 IU/g (Umsza-Guez et al., 2011).

3.5. Degradation of melanoidins by A. awamori

3.5.1. SSF experiment

As shown in Table 3, water-extracted melanoidins with MW of 10–100 kDa decreased from 5.29 g/100 g to 1.38 g/100 g after fermentation, and those with MW of >100 kDa was lowered from 1.85 g/100 g to 1.12 g/100 g after SSF. Alkaline-extracted melanoidins showed a greater reduction after fermentation: 10–100 kDa melanoidins dropped by 69% (from 7.27 g/100g to 1.95 g/100 g), and > 100 kDa melanoidins reduced by 85% (from 5.29 g/100 g to 0.78 g/100 g). Melanoidins with different MW and solubility characteristics all showed a reduction with a total loss of 73.5% after SSF by *A. awamori,* indicating their breakdown into smaller molecules and utilized as an energy source by *A. awamori*.

Table 3
The content of high molecular weight melanoidins separated by ultrafiltration in water and alkaline extracts of DSG before and after fermentation by *A. awamori*.

Fermentation period (days)	Extraction way	Melanoidins (10–100 kDa, g/100 g DSG)	Melanoidins (>100 kDa, g/100 g DSG)
0	Water extraction	5.29 ± 0.27	1.85 ± 0.87
5		1.38 ± 0.22	1.12 ± 0.21
0	Alkaline extraction	7.27 ± 0.68	5.29 ± 0.60
5		1.95 ± 0.20	0.78 ± 0.12

The results were shown as mean ± standard deviation (SD) (n=3).

The degradation of melanoidins by fungi such as *Aspergillus*, *Rhizopus,* and *Penicillium* has been found in many studies, but they mainly focused on the distillery effluent (Rani and Pal, 2013). Our data showcases the degradation of food-derived melanoidins by *A. awamori,* also providing an alternative for the elimination of melanoidin-induced environmental contamination. To further study this effect, melanoidins were isolated from DSG and used in an SmF process as the only carbon and nitrogen sources. *A. awamori* and *A. oryzae* were tested to evaluate their ability for melanoidin degradation.

3.5.2. SmF experiments

As shown in Fig. 5, *A. awamori* and *A. oryzae* reduced melanoidin browning and exhibited significant biomass growth in SmF. During the first three days of SmF, the browning of melanoidins decreased steadily, reaching its lowest point on the third day, with a decolorization of 24.4% by *A. awamori* and 12.4% by *A. oryzae* compared to time zero, respectively (Fig. 5a). *A. awamori* presented a stronger decolorization ability than *A. oryzae*. The browning of melanoidins in the SmF medium gradually increased from day 3 to day 5, likely due to fungal metabolic activities producing brown compounds (see the discussion in section 3.3). By day 5, the browning of the fermented melanoidins was still lower than that of the unfermented sample, indicating that degradation was still effective. The fermentation of 3 days would be an optimal time point for harvesting considering achieving maximum melanoidin degradation.

The ability of fungi to decolorize melanoidins is related to the type of fungi and the source of melanoidins. Enhancing the melanoidin culture medium with suitable nutrients facilitates the decolorization of melanoidins. Shayegan et al. (2005) reported the maximum decolorization of 85% to distillery wastewater using isolated *Aspergillus* species under optimal conditions. A possible mechanism of melanoidin decolorization is the oxidation effect by enzymes, typically including laccase, manganese peroxidases, and lignin peroxidases (Rani and Pal, 2013). Detudom et al. (2019) genetically engineered *Bacillus subtilis to* secrete laccases to degrade soy sauce melanoidins. After 7 days of cultivation, the cell-free supernatant of the engineered strains achieved a maximum decolorization capacity of 70.4% of synthetic soy sauce melanoidins – made from 10% xylose and soy peptone.

As shown in Fig. 5b, fungal biomass increased during the fermentation process. The rapid growth period of fungi occurred in the first two days, and the stable period was then reached with a maximum biomass 2.5 g/L for *A. awamori* and 1.5 g/L for *A. oryzae*. These results suggested that melanoidins from DSG can be utilized as a carbon source by *A. awamori* and *A. oryzae* and converted into mycelial biomass.

The fermentation products were further investigated to understand the degradation and metabolism of melanoidins by fungi. Glucose, followed by arabinose and xylose, predominates in alkaline extracts of DSG melanoidins (Yang et al., 2023). Fig. 5c presents a significant rise in free monosaccharides, particularly arabinose, glucose, and xylose, in the melanoidin medium after 3 days of *A. awamori* fermentation, compared to the control. This result indicated that melanoidins were degraded by *A. awamori* and simple sugars were released for microbial growth during this phase. The lower amount of glucose than arabinose left in media may be due to the type of enzymes released or the utilization preference of fungal for glucose over other sugars (Blandino et al., 2002). The higher level of monosaccharide in media by *A. awamori* compared to *A. oryzae* proved that *A. awamori* may have a stronger ability to metabolize melanoidins. Following a further 2 days of fermentation, the free monosaccharides experienced a decrease in concentration, as the fungal metabolism used the sugar as feed.

The metabolic activity of *A. awamori* and *A. oryzae* on melanoidins was confirmed by changes in total protein content over time (Fig. 5d). Protein, one of the chemical components of melanoidins, showed a

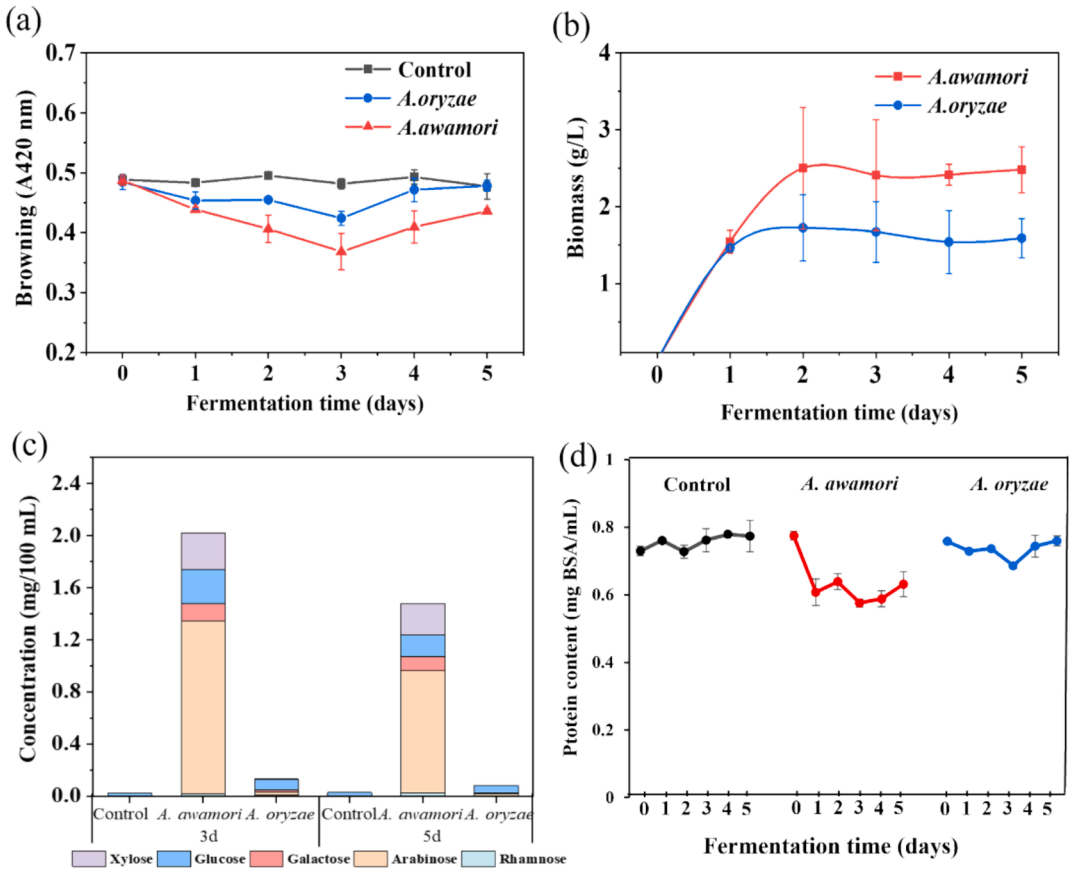

Fig. 5. Changes in the browning index (Abs 420 nm) (a), biomass (b), free monosaccharides (c), and protein (d) concentration during SmF of DSG melanoidins.

downward trend in the first 3 days, which could stem from the activity of proteases from *A. awamori* and *A. oryzae,* as both species are known to generate proteases (Lin et al., 2013). As fermentation progressed to day 5, protein levels increased in the media. Considering the stable biomass (Fig. 5b) and a decrease in monosaccharide concentration (Fig. 5c) from day 3 to 5, it is likely that fungi stopped growing and consumed sugars as energy for metabolite production, like synthesizing new proteins, thereby increasing the protein content in the medium.

3.5.3. The comparison of SSF and SmF by A. awamori

A. awamori exhibited varied fermentation performances and effects in SSF and SmF. For instance, *A. awamori* biomass consistently increased over 5 days in SSF, whereas it peaked on the second day in SmF. The color in SSF continued to darken, while in SmF, the substrate initially deepened and then lightened. Soluble protein also exhibits different content changes in SSF and SmF. These variations can be attributed to the differences in substrate and environmental conditions. In SmF, melanoidins were used as the sole carbon source, limiting the nutrients available to *A. awamori*. In contrast, SSF of DSG provides a more diverse nutrient environment for *A. awamori*, likely promoting broader metabolic activity and functional gene expression such as cellulase and hemicellulose genes, protease genes, and amylase genes (Shauna and Richard, 2010). A broader gene expression further facilitates better nutrient breakdown and absorption, causing the difference in SSF and SmF.

4. Conclusions

DSG can be upcycled to fungal biomass through fermentation by *A. awamori*. The fermentation process increases the concentration of water-soluble proteins, phenolic compounds, and colored compounds in DSG extracts. Moreover, *A. awamori* effectively degrades the melanoidins present in DSG. The SmF of DSG melanoidins by both *A. awamori* and *A. oryzae* added evidence to these findings. This study presents a new strategy for the degradation of food melanoidins and the upcycling of melanoidin-rich agro-industrial waste through SSF by food-grade fungi. Future research should focus on the specific enzymes responsible for melanoidin degradation and optimizing the fermentation process.

CRediT authorship contribution statement

Shiqi Yang: Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Sultan Arslan-Tontul:** Writing – review & editing, Conceptualization. **Vincenzo Fogliano:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Melania Casertano:** Writing – review & editing, Conceptualization. **Wenlai Fan:** Supervision, Funding acquisition, Conceptualization. **Yan Xu:** Resources. **Yao Nie:** Supervision, Conceptualization. **Arnau Vilas-Franquesa:** Writing – review & editing, Validation, Supervision, Conceptualization.

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.biortech.2024.131817.

Data availability

Data will be made available on request.

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