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Research Article

Optimization of solid-state fermentation for protein enrichment in rice protein residue and corn germ powder using edible mushroom mycelium

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Abstract: Rice protein residue (RPR) and corn germ meal (CGM) are industrial by-products that are commonly applied as animal feed with low economic benefits. In order to develop a new approach for the resource utilization of grain protein residue, this study converted grain protein residue into edible mushroom protein through solid-state fermentation (SSF). To increase the biological efficiency of biotransformation, this study used Box Behnken design, combined with single factor experiments and response surface methodology, to optimize the SSF process parameters of edible mushroom mycelium. Optimal fermentation conditions were established considering both protein content and operational feasibility: RPR to CGM ratio of 4:1, utilizing the *Pleurotus ostreatus* strain, a fermentation duration of 14.5 days, a solid-to-liquid ratio of 1:0.8, and a loading capacity of 65.59 g. Fermentation under these optimized conditions yielded 73.34 g of protein per 100 g of RPR/CGM blend, which is 98.71% of the predicted value and represents a 1.28-fold increase from the initial protein content of 56.96 g/100 g. The amino acid evaluation results showed that the total amino acid content of the fermented protein residue increased by 12.88%, with a significant increase in the concentrations of glutamic acid and aspartic acid, which increased by 19.53% and 24.27%, respectively. In addition, the amino acid ratio coefficient score (SRC) and nutritional index (NI) were slightly higher, indicating a more balanced proportion of essential amino acids after fermentation. Additional research on the physicochemical properties of the protein residue post-fermentation revealed that the emulsifying capacity improved by 3.5% compared to the non-fermented sample. Edible mushrooms are a promising method for converting RPR and CGM into high-protein raw materials.

Keywords: rice protein residue; corn gem meal; edible mushroom mycelium; solid state fermentation; protein

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1 Introduction

Rice protein residue (RPR) is a byproduct of the production of monosodium glutamate, starch sugar, and organic acids. In the process of producing starch sugar, when 7 t of rice are used to make starch sugar, 1 t of rice residue will be produced. The starch sugar production in China in 2022 is 16.88 million t. The annual production of rice residue in China is estimated to be 2.41 million t based on the production of starch sugar. The content of RPR-based protein is as high as 70%; however, its solubility is poor, and it is directly disposed of as animal feed, resulting in a significant waste of protein resources[1]. Meanwhile, it is estimated that the world's corn production exceeded 11.363 billion t (MMT) from September 2021 to August 2021, with approximately 31.7% (3.603 MMT) produced in the United States, followed by China (2.606 MMT) and Brazil (1.090 MMT) (https://www.fas.usda.gov). Chinese corn is mainly used as feed (65.77%) and for industrial consumption (27.30%). China consumes approximately 71.4 million t of corn annually for industrial consumption, and based on an average embryo extraction rate of 7%, the corn germ production reaches 5 million t. The theoretical yield of corn germ oil is approximately 1.72–2.79 million t, and the annual production of corn germ meal (CGM) ranges from 3.28 million t to 2.21 million t. The by-products of CGM oil pressing and extraction, except for a decrease in fat content, retain most of the nutrients. Depending on the oil pressing method and corn variety, the crude fat content of CGM ranges from as low as 0.20% to as high as 12.09%. The crude protein content in CGM is generally between 10.13% and 24.79%[2, 3], making it a high-quality protein source. The crude fiber content of CGM is relatively high, with a

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variation range of 2.18%–21.92%[2,4], which is four times the fiber content of corn. However, the research and application of CGM are still mainly limited to animal feed, highlighting the gap in its potential for human food innovation[5]. The use of RPR and CGM substrates in mushroom fermentation to produce high-protein food ingredients is an efficient, sustainable, and health-conscious method.

With the projected growth of the global population to 9–10 billion by 2050 and an anticipated rise in meat demand to 455 million t, protein resources have become a vital concern[6]. Microbial protein, notably more productive and biologically efficient than animal protein, offers a promising avenue to mitigate the health crises facing humanity and the Earth[7]. Fermenting plant protein using fungi, particularly from agricultural waste, can enhance its bioavailability and overall content[8,9], positioning microbial protein as a preferred alternative in future protein products (dairy analogues, plant-based meat products, high protein blended products, etc.), and providing varied options for expanding vegetarian populations and health-conscious consumers.

Fermentation is a longstanding food processing technique , and solid-state fermentation (SSF) of edible mushrooms usually uses agricultural waste with high cellulose content as the culture medium, such as wheat bran[10], rice husk[11], cottonseed husk[12], etc. However, only a small number of studies had utilized edible fungi to ferment grain protein residues. For example, Wang *et al.*[9] utilized SSF of soybean meal using edible mushroom mycelium to enhance its nutritional, antioxidant capacity, and physicochemical properties. Previous studies, such as those by Ou *et al.*[6], had found that fermenting soybeans with mixed strains could simulate plant-based meat, yielding improvements in color, texture, and flavor. Fungal SSF in particular had been shown to transform plant proteins into fungal proteins, thus jazzing up the proteins' quality and expanding the amino acid profile. For instance, Clark *et al.*[13] successfully used shiitake mushroom mycelium to ferment a blend of pea and rice proteins, enhancing the digestibility, nutritional value, and sensory attributes of the mix. Nevertheless, the research on high-protein products (new functional foods, such as plant-based foods represented by plant-based meat), especially via SSF of edible mushrooms, is nascent but harbors significant promise.

This experimental investigation will employ edible fungi for the SSF of grain residues, with a focus on identifying suitable strains for RPR and CGM fermentation and optimizing fermentation process parameters through both single-factor experiments and response surface methodology. And the amino acid content of grain protein residue would be determined, and its nutritional value would be evaluated. Additionally, the study will delve into the physicochemical properties of the fermented residues—specifically, their water retention, oil absorption, emulsifying capacities, and emulsifying stability—in an effort to provide technical insights for the comprehensive utilization of grain resources and the advancement of fermented grain protein residue products.

2 Materials and methods

2.1 Material

RPR with a protein content of 67% was obtained from Hangzhou Zixiang Sugar Industry Co., Ltd. (Hangzhou, China). CGM, comprising 14% protein, was sourced from Muyang County Huafeng Feed Development Co., Ltd. (Suqian, China). The fungus *Lentinus edodes* L808 (Le) was procured from the Quzhou Academy of Agricultural Sciences (Quzhou, China). Strains of *Pleurotus ostreatus* ACCC 50476 (Po), *Pleurotus abalonus* ACCC 52627 (Pa), *Flammulina velutipes* ACCC 51540 (Fv), and *Ganoderma lucidum* ACCC 50621 (Gl) were acquired from the China General Microbiological Culture Collection Center (Beijing, China). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, 99%), potassium dihydrogen phosphate (KH_2PO_4, 99.5%), sulfuric acid (H_2SO_4, 99.7%), hydrochloric acid (HCl, 12 mol/L), and sodium hydroxide (NaOH, 96%) were all supplied by China National Pharmaceutical Group Co., Ltd. (Shanghai, China). Potato dextrose broth (PDB), produced by Beijing Aobo Xing Biotechnology Co., Ltd., was used for the growth of fungal cultures. All additional chemicals employed in this study were of analytical grade.

2.2 Solid state fermentation process flow

Fermentation strain cultivation: Preserved strains were inoculated onto PDB medium and incubated at a constant temperature of 25 °C (LRH-250, Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) for 6 days in preparation for further use.

Liquid strain activation: Strains transferred from the PDB medium into a liquid medium were cultured at a constant temperature of 25 °C and 180 r/min for a period of 4–12 days to obtain an activated edible mushroom seed liquid.

Fermentation process, as shown in Fig. 1. Raw materials were mixed with distilled water and placed into fermentation bottles; 0.2% magnesium sulfate and 0.1% potassium dihydrogen phosphate were then added, thoroughly stirred, and subsequently sterilized using high-pressure steam at 121 °C for 30 min. Following sterilization, 10 mL of the edible mushroom seed liquid was introduced into the bottles for SSF, which were then incubated at 25 °C in the dark for 15 days. Post-incubation, the fermentation products were dried in an air-circulating oven (DHG-9070A, Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) at 50 °C for 24 h. To achieve uniform particle size, the dried material was ground to a powder and passed through an 80-mesh screen to produce the fermented protein powder[9]. All experimental conditions were replicated in triplicate.

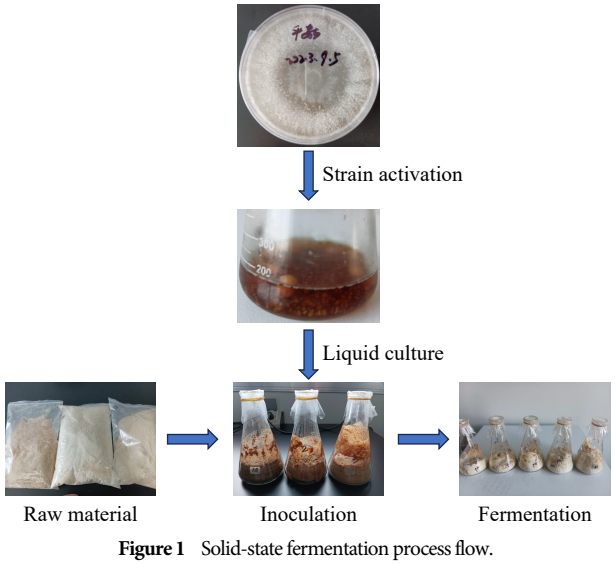

Figure 1 Solid-state fermentation process flow.

2.3 Screening of fermentation strains

The selection of strains suited for growth on RPR/CGM comprised testing with Gl, Fv, Le, Po, and Pa. The fermentation conditions entailed the following: an RPR/CGM ratio of 4:1; an initial solid-liquid ratio of 1:1; the loading capacity was 25 g/250 mL (protein residue mass/conical flask volume); the inoculation volume of the edible mushroom at 10% of the loading capacity; a constant cultivation temperature of 25 °C; and a culture duration of 15 days. Protein content served as the primary evaluation metric.

2.4 Determination of RPR to CGM ratio

The study utilized varying proportions of an RPR and CGM mixture as the culture substrate, with RPR constituting 60%, 80%, and 100%, respectively. Preserving the initial solid-liquid ratio at 1:1, the loading capacity was 50 g/500 mL (protein residue mass/conical flask volume). the inoculation amount for Po and Gl was fixed at 10% of the loading capacity, with a controlled cultivation temperature of 25 °C over a 15-day period. Observation of the mycelium growth condition and quantification of its protein content were conducted to identify the optimum SSF substrate.

2.5 Single factor experiment

2.5.1 Determination of loading capacity

A 500 mL Erlenmeyer flask was utilized for fermentation, testing loading capacities of 25, 50, 75, 100, and 125 g, together with the optimal fermentation strain identified in Section 2.3. The chosen RPR/CGM ratio from Section 2.4 was used, maintaining an initial solid-liquid ratio of 1:1, an inoculation amount of 10%, a cultivation temperature of 25 °C, and a time period of 15 days. Protein content served as the performance indicator.

2.5.2 Determination of cultivation time

Cultivation times of 3, 6, 9, 12, and 15 days were evaluated. The best fermentation strain as determined in Section 2.3, the optimal RPR/CGM ratio from Section 2.4, and the preferable loading capacity from Section 2.5.1 were used. The experimental setup was consistent with an initial solid-liquid ratio of 1:1, an inoculation amount of 10%, and a cultivation temperature of 25 °C. Protein content remained the key evaluation metric.

2.5.3 Determination of solid-liquid ratio

A range of initial solid-liquid ratios—1:0.6, 1:0.8, 1:1, 1:1.2, and 1:1.4—were tested for fermentation. This phase incorporated the optimal fermentation strain from Section 2.3, the optimal loading capacity from Section 2.5.1, the best RPR/CGM mix ratio from Section 2.4, and the optimal cultivation time from Section 2.5.2. An inoculation rate of 10% and a stable cultivation temperature of 25 °C were maintained throughout. Once again, protein content was used to gauge the results.

2.6 Response surface optimization experiment

Building upon the single-factor experiments, response surface methodology—guided by the Box-Behnken design's central composite arrangement—was employed, focusing on three independent variables: loading capacity, the solid-liquid ratio, and cultivation time. This approach structured a three-factor, three-level experimental design intended to refine the fermentation conditions, utilizing the protein content of the fermented residue as the response variable. The experimental design, data processing, and model development were performed using Design-Expert software, version 13.

2.7 Determination of protein content

The crude protein content was determined following the AOAC 960.52 semi-micro Kjeldahl method, applying a conversion factor of 6.25. Briefly describe as follows: Weigh 0.2 g of solid sample into a digestion tube, add 0.4 g of copper sulfate, 6 g of potassium sulfate, and 20 mL of sulfuric acid, and then put it into a digestion furnace for digestion. After the temperature of the digestion furnace reaches 420 °C, continue digestion for 1 h. At this time, the liquid in the digestion tube is green and transparent. After cooling, add 50 mL of water and perform automatic liquid addition and distillation on an automatic Kjeldahl nitrogen analyzer. Then titrate with HCl (0.05 mol/L) and record the data.

2.8 Determination of amino acid content and nutritional value of grain protein residue

Protein residue before fermentation (PRBF), sterilized protein residue (SPR), and fermented protein residue (FPR) were weighed and added to the hydrolysis tube, respectively. Add 10 milliliters of 1:1 analytical-grade hydrochloric acid (approximately 6 mol/L), blow nitrogen into the tube for 30 s, seal, and place it in an oil bath at 110 °C for 22–24 h for hydrolysis. After hydrolysis, cool to room temperature, filter through a 0.45 μm membrane into a 50 mL volumetric flask, and make up for the volume. After reaching a constant volume, take 2 mL of the sample and place it on a rotary evaporator at 45 °C for deacidification, and let it dry until there is a small amount of solid or residue at the bottom of the bottle. Add 2 mL of sample buffer to dissolve completely, filter through a 0.45 μm filter, and analyze using an automatic amino acid analyzer (Biochrom 30+, UK). According to the nutritional assessment calculation method of the Food and Agriculture Organization/World Health Organization (FAO/WHO), calculate the essential amino acid (EAA) score, essential amino acid index (EAAI), amino acid score (AAS), amino acid ratio coefficient score (SRC), nutritional index (NI), essential amino acid to total amino acid ratio (EAA/TAA), and protein efficiency ratio (PER) of protein residual samples before and after fermentation. Then refer to the relevant formulas of Chavan *et al.*[14] as follows.

The EAA/TAA were calculated as follow:

$$\begin{aligned}&\mathrm{EAA/TAA}\ (\%) = \\ &(\mathrm{Met}+\mathrm{Cys}+\mathrm{Phe}+\mathrm{Tyr}+\mathrm{Thr}+\mathrm{Ile}+\mathrm{Val}+\mathrm{Leu}+\mathrm{Lys}+\mathrm{His})/ \\ &(\mathrm{Glu}+\mathrm{Gly}+\mathrm{Asp}+\mathrm{Ser}+\mathrm{Ala}+\mathrm{Pro}+\mathrm{Arg}+\mathrm{Met}+\mathrm{Cys}+ \\ &\mathrm{Phe}+\mathrm{Tyr}+\mathrm{Thr}+\mathrm{Ile}+\mathrm{Val}+\mathrm{Leu}+\mathrm{Lys}+\mathrm{His})\times 100\end{aligned} \quad (1)$$

The AAS was calculated according to the method[15] as follow:

$$\mathrm{AAS}\,(\%) = \frac{\text{EAA in sample protein}}{\text{EAA in reference protein pattern}} \times 100 \quad (2)$$

The SRC indicated the degree to which a food's amino acid deviates from the standard pattern:

$$\mathrm{SRC} = 100 - \sqrt{\sum_{i-1}^{n} \frac{\left(\mathrm{RC}_i - \overline{\mathrm{RC}}\right)^2}{\frac{(n-1)}{\overline{\mathrm{RC}}}}} \times 100 \quad (3)$$

$$RC_i = \frac{AAS_i}{\overline{AAS}} \quad (4)$$

where, n is the number of EAAs; RC_i and AAS_i stand for the ratio coefficient of EAAs and the score of the EAAs; $\overline{AAS}$ and $\overline{RC}$ mean the average value of the score of the EAA and RC in the tested food protein, respectively. The reference protein used was the EAA pattern from Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO)/United Nations University (UNU) [16]

The nutritional quality of protein powder before and after fermentation was evaluated based on their EAA profile. The EAAI and NI were calculated as follows[15]:

$$EAAI(\%) = \sqrt[n]{\frac{Lys_{I\ sample}}{Lys_{I\ reference}} \times \frac{Tyr_{sample}}{Tyr_{reference}} \times \cdots\cdots \times \frac{His_{n\ sample}}{His_{n\ reference}}} \times 100 \quad (5)$$

$$NI = \frac{EAAI \times Protein\ (\%)}{100} \quad (6)$$

Where n is the number of EAAs included. The sample and reference mean the test protein and reference protein, respectively. The reference protein used was the EAA pattern from FAO/WHO/UNU[16].

The PER values of grain protein residue were calculated by amino acid compositions with the following equations:

$$PER1 = -0.684 + 0.456 \times (Leu) - 0.047 \times (Pro) \quad (7)$$

$$PER2 = -0.468 + 0.454 \times (Leu) - 0.105 \times (Try) \quad (8)$$

$$PER3 = -1.816 + 0.780 \times (Leu) - 0.944 \times (Tyr) + 0.435 \times (Met) + 0.211 \times (His) \quad (9)$$

2.9 Physical and chemical characteristic analysis

2.9.1 *Determination of water holding capacity (WHC)*

The WHC of the protein residue before and after fermentation was determined using the procedure described by Zheng *et al.*[17]. A sample of 0.5 g of protein residue was thoroughly mixed with 50 mL of distilled water, allowed to rest at room temperature for 18 h, and then centrifuged at 10,000 r/min for 20 min. The supernatant was discarded, and the mass of the water-absorbed sample was weighed.

$$WHC(g/g) = \frac{(m_1 - m_o)}{m_o} \quad (10)$$

where m_1 was the mass of the sample after water absorption (g), and m_o was the initial mass of the sample (g).

2.9.2 *Determination of oil holding capacity (OHC)*

The procedure by Zheng *et al.*[17] was followed to determine the oil-holding capacity of protein residues before and after fermentation. A 0.5 g sample of protein residue was blended with 30 mL of soybean oil and left to stand at room temperature for 18 h before being centrifuged at 10,000 r/min for 20 min. Following centrifugation, the supernatant was discarded, and the residue was weighed.

$$OHC(g/g) = \frac{(m_1 - m_o)}{m_o} \quad (11)$$

where m_1 was the mass of the sample after oil absorption (g), and m_o was the initial mass of the sample (g).

2.9.3 *Determination of emulsifying capacity (EC) and emulsion stability (ES)*

The EC and ES of protein residues before and after fermentation were assessed as per the method outlined by Chan *et al.*[18]. Add 3.5 g of protein residue to 50 mL of distilled water and homogenize at 8,000 r/min (TG16-WS, Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Hunan, China) for 30 s. Subsequently, add 50 mL of soybean oil and homogenize for another 30 s. Divide the mixture into two 50 mL centrifuge tubes and centrifuge at 1,100 × g for 5 min. EC was ascertained by dividing the height of the emulsion layer by the total height of the mixture in the centrifuge tube. After the initial EC measurement, the emulsion was incubated in a water bath at 80 °C for 30 min and re-centrifuged at 1,100 × g for 10 min. ES was calculated using the same procedure as EC.

2.10 Statistical analysis

Three replicates of each sample were prepared. All analyses were conducted in triplicate. The data are presented as the mean ± standard deviation (SD). Charts were created using Origin 2021 (OriginLab Corp., Northampton, MA, USA). Statistical analyses were performed using the Duncan test and SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). A P-value of less than 0.05 was considered to indicate statistical significance.

3 Results and analysis

3.1 Screening results of strains

To identify suitable edible fungi for growth on protein residue, this study investigated five different edible mushroom species. Using unfermented protein residue as a control and protein content as the evaluation criterion, these fungal strains were preliminary screened. The results, presented in Fig. 2A, indicate a significant increase in protein content in FPR samples. Notably, the strains Po and Gl showed superior performance during fermentation, with protein content surpassing 70%. Relative to the original protein residue, protein content increased by 28.37% and 25.04% for Po and Gl, respectively. In this experiment, the protein content of Po (72.27%) was slightly higher than that of Gl (70.39%), so Po was chosen as the fermentation strain. Such fermentation processes can elevate protein content in substrates, aligning with findings from previous studies. For instance, Li *et al.*[19] observed a 1.93-fold increase in protein content using SSF of tartary buckwheat with shiitake mushrooms. Similarly, Asensio-Grau *et al.*[20] reported a 23.00% boost in protein content by employing edible mushrooms for the SSF of lentils. The observed increase in protein content may be due to microbial growth, which consumes nitrogen and carbon sources and produces mycelial protein, resulting in a relative or absolute increase in protein content. To sum up, this study confirms that SSF of edible fungi can increase the protein content of the substrate, which can be used to develop new high-protein food raw materials.

3.2 Determination of the ratio of RPR to CGM

The growth and metabolic activities of microorganisms are contingent upon the availability of carbon and nitrogen sources, which supply the energy and facilitate biosynthesis processes. The choice of growth medium plays a key role in producing edible

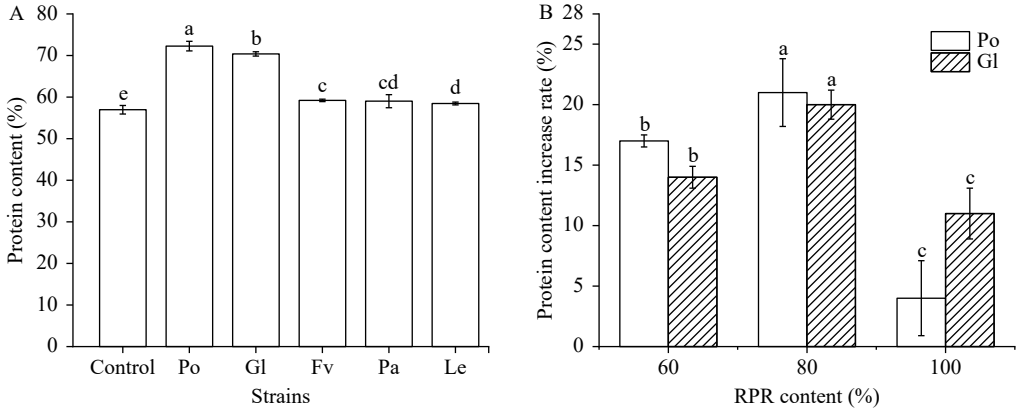

Figure 2 Protein content of fermented protein residue from various strains (A) and protein improvement rate of different RPR contents (B).

mushrooms by providing the necessary nutrients for mycelial development[21]. The protein content of RPR (67% protein) used in this experiment is 4.79 times that of CGM (14% protein). The RPR proportion in the solid matrix influenced not only the growth of the edible fungi but also the protein content of the end fermentation product. The aim of this study is to develop a novel high-protein food ingredient as a substitute protein for meat products. Therefore, while ensuring the normal growth conditions of edible mushrooms, high-protein ingredients should be used as much as possible. As depicted in Fig. 2B, with an RPR addition ratio of 80%, the protein content for strains Po and Gl exhibited an average increase of 20%. This enhancement was significantly more pronounced at RPR addition ratios of 60% and 100%. Consequently, for this study, a SSF substrate with a 4:1 RPR to CGM ratio was chosen to optimally promote the growth of the edible fungi and raise the protein content in the resulting protein powder.

3.3 Analysis of single-factor results

The influence of loading capacity, solid-liquid ratio, and cultivation time on the protein content of the protein residue was examined, with protein content functioning as the evaluation index.

3.3.1 Effect of loading capacity on protein residue fermentation

Microorganism growth and metabolism are impacted by their loading capacity within a fixed container. Optimal loading ensures sufficient oxygen and nutrient supply to meet the strain's requirements during fermentation[22]. As depicted in Fig. 3A, the effect of varying loading capacity on protein content was comparatively minor, exhibiting an initial decrease, followed by an increase, and then another decrease as the loading capacity increased. At a reduced loading capacity, the ample oxygen supply supported vigorous mycelium growth, with a 25 g/500 mL loading yielding a higher protein content. In contrast, a loading condition of 50 g/500 mL led to nutrient depletion in the culture medium, consequently hindering mycelium growth. With loading capacities of 75–125 g/500 mL, the relative content of mycelium in the culture was low, resulting in a decrease in overall protein content. In addition, the time required for mycelium to fill the fermentation container increased with the increase of loading capacity. Particularly at 75–125 g/500 mL loading, the required fermentation time was extended, impacting efficiency. Therefore, for better production efficacy and energy conservation, a loading capacity of 50 g/500 mL for the fermentation substrate was recommended.

3.3.2 Effect of solid-liquid ratio on protein residue fermentation

Moisture content is pivotal for the growth of microorganisms and the production of metabolic products during SSF[23]. As illustrated in Fig. 3B, the protein content initially increased, then plateaued, and finally decreased with a descent in the solid-liquid ratio. A relatively high protein content in RPR was maintained within an initial moisture range of 80%–120%. Conversely, an overly high or low moisture content (e.g., 60%) hindered mycelial growth, resulting in reduced protein content. Insufficient moisture may stimulate sporogenesis in microorganisms, while excessive moisture can restrict oxygen supply, impede mycelial extension (Fig. S1), and raise the risk of bacterial contamination[24]. Consequently, this study selected a solid-liquid ratio of 1:0.8 as the optimal initial moisture condition for the fermentation process.

3.3.3 Effect of cultivation time on protein residue fermentation

The nutrient concentrations available to microorganisms dynamically decrease over the course of fermentation, significantly affecting microbial biomass[25]. Microorganisms typically experience senescence and autolysis, discharging enzymes to degrade their own secondary metabolites. Fig. 3C showed the effect of different fermentation times on protein content; a swift surge in protein levels initially occurred, which then progressively decelerated. After 9 days, the protein content's growth rate diminished, while during periods of 12–15 days of fermentation, it attained higher levels. This trend can be attributed to the depletion of culture medium nutrients as the fermentation period lengthens, yielding insufficient sustenance for fungal strains. The resulting strain degradation and the dampened growth of mycelium consequently mitigate the rate of increase in protein content.

3.4 Response surface optimization of the protein residue fermentation process

Taking cues from the single-factor experiment and guided by the principles of the Box-Behnken central composite design, a three-factor, three-level experiment was conducted. Three independent variables—protein residue fermentation time, solid-liquid ratio, and loading capacity—were tested against the protein content of the FPR to refine the fermentation conditions. The experimental design and its outcomes are listed in Table 1.

3.4.1 Model establishment and significance analysis

With the aid of Design-Expert software (Version 13), quadratic polynomial regression analysis was carried out on the data from Table 1, yielding the following regression equation between fermentation time (*A*), solid-liquid ratio (*B*), loading capacity (*C*),

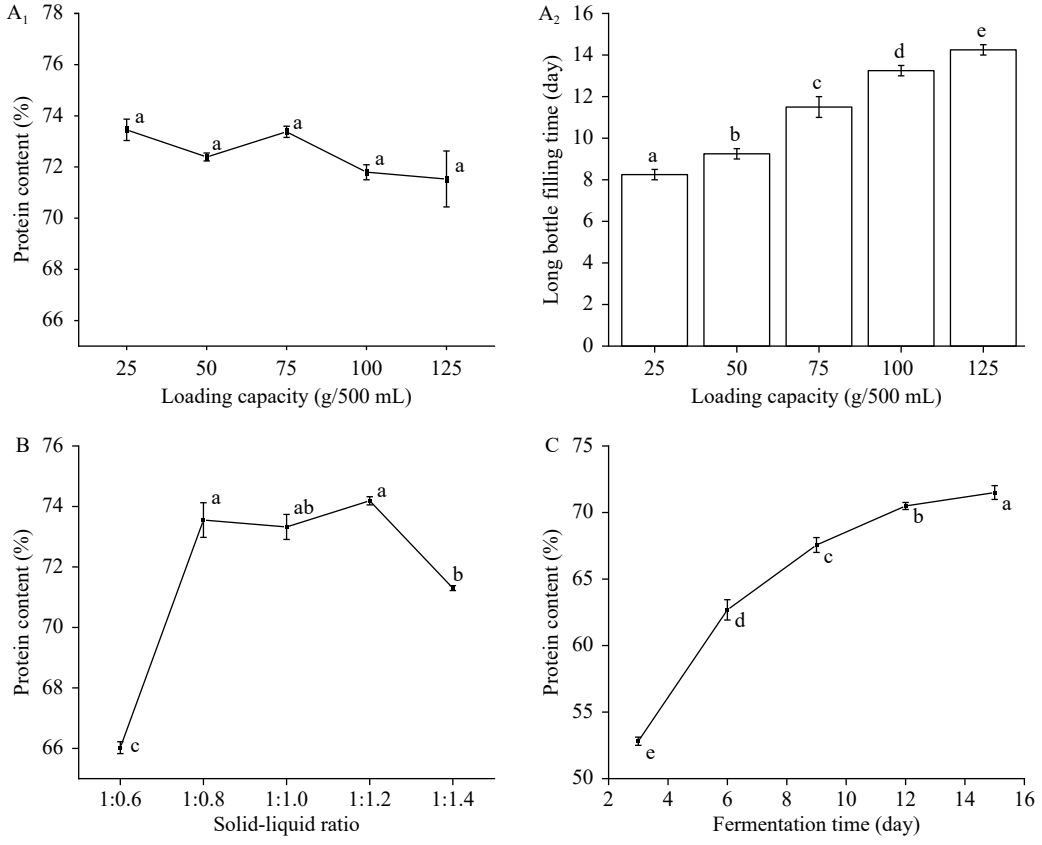

Figure 3 Protein content of fermented protein residue with different loading capacity (A), solid-liquid ratios (B), and fermentation time (C).

Table 1 Response surface optimization experimental design and results

Experiment number	*A* Fermentation time (day)	*B* Solid-liquid ratio	*C* Loading capacity (g)	*Y* Protein content (g/100 g)
1	0	0	0	70.96
2	0	0	0	71.68
3	1	1	0	70.45
4	0	−1	1	66.80
5	0	1	−1	69.91
6	−1	0	1	70.60
7	1	−1	0	74.07
8	0	0	0	70.68
9	0	0	0	71.22
10	−1	−1	0	67.64
11	1	0	−1	74.24
12	−1	1	0	71.57
13	0	0	0	70.60
14	0	1	1	68.48
15	0	−1	−1	69.67
16	1	0	1	71.51
17	−1	0	−1	71.74

Note: In the fermentation time, 0 represents 10.5 days, 1 represents 15 days, and −1 represents 6 days. In the solid-liquid ratio, 0 represents 1:1, 1 represents 1:1.4, and −1 represents 1:0.6. In the loading capacity, 0 represents 87.5 g, 1 represents 125 g, and −1 represents 50 g.

and protein content (Y):

$$Y = 71.03 + 1.09A + 0.28B - 1.02C - 1.89AB - 0.40AC + 0.36BC + 1.60A^2 - 1.71B^2 - 0.61C^2$$

An analysis of variance (ANOVA) was applied to the regression model. The ANOVA results and the coefficients of the fitted model for protein content are presented in Table 2. The mismatch test of the regression equation was found to be nonsignificant (P = 0.367,8), suggesting minimal interference from unknown factors in the experimental outcomes, while the model fit was significant ($P < 0.01$). The determination coefficient R^2 of the model was 0.973,5, and the adjusted judgment coefficient (R^2_{Adj}) was 0.939,4, reflecting a good model fit to the experimental data and accurately capturing the relationship between protein content and the three tested variables: fermentation time, solid-liquid ratio, and loading capacity. Consequently, the derived regression equation is capable of effectively predicting the changes in protein content under various fermentation parameters. Considering the P-values, the quadratic term for the loading capacity (C^2) was found to significantly affect the outcome ($P < 0.05$), while the primary terms (A and C) and the interaction and quadratic terms (AB, A^2, and B^2) had a highly significant impact ($P < 0.01$). Based on the F-values, the extent of influence of the three factors on the protein content of the residues was fermentation time (A) > loading capacity (C) > solid-liquid ratio (B).

3.4.2 Analysis and optimization of different factors and their interactions

Design-Expert software, version 13, was utilized to construct three-dimensional response surface plots and contour maps based on the regression model, facilitating the analysis of the interaction effects between different factors on protein content.

3.4.2.1 Effect of the interaction between loading capacity and solid-liquid ratio on protein content

Figures 4A and 5A illustrated the impact of the interaction

between loading capacity and solid-liquid ratio on protein content. Observations indicated no significant interaction effect on protein content attributed to loading capacity paired with solid-liquid ratio, aligning with the insights from Table 3. At lower loading capacities, the protein content first increased and subsequently decreased as the solid-liquid ratio grew.

3.4.2.2 Effect of the interaction between loading capacity and fermentation time on protein content

The impact of the interaction between loading capacity and fermentation time on protein content is depicted in Figs. 4B and 5B. There appeared to be no significant interaction effect on protein content regarding loading capacity and fermentation time, which concurs with the findings presented in Table 2. As suggested by Fig. 3A, an increase in loading capacity led to a corresponding extension of the time needed for mycelium to fully colonize the culture bottle, theoretically implying a significant relationship between loading capacity and fermentation time would be expected. Nonetheless, the growth and metabolic activities of mycelium are dependent on adequate nitrogen sources. During the initial fermentation stages, protein consumption in the substrate outpaces mycelial protein biosynthesis. Consequently, at higher loading capacities, protein content tended to first diminish and then increase as fermentation progressed. Conversely, under low loading capacity, protein content consistently rose as fermentation time lengthened.

3.4.2.3 Effect of the interaction between solid-liquid ratio and fermentation time on protein content

The combined effect of solid-liquid ratio and fermentation time was found to significantly influence protein content. As demonstrated in Figs. 4C and 5C, this interaction conforms to the findings detailed in Table 2. At lower solid-liquid ratios, an increase in fermentation time consistently led to higher protein content. Conversely, at elevated solid-liquid ratios, protein content initially declined before rising in tandem with fermentation time.

Table 2 Significance test and analysis of variance for protein content regression model

Source	Sum of squares	df	Mean square	*F*-value	*P*-value	Significant
Model	57.4	9	6.38	28.57	0.000,1	**
A	9.48	1	9.48	42.48	0.000,3	**
B	0.617,7	1	0.617,7	2.77	0.140,2	
C	8.35	1	8.35	37.39	0.000,5	**
AB	14.26	1	14.26	63.88	<0.000,1	**
AC	0.631,6	1	0.631,6	2.83	0.136,5	
BC	0.519,4	1	0.519,4	2.33	0.171	
A^2	10.84	1	10.84	48.55	0.000,2	**
B^2	12.24	1	12.24	54.84	0.000,1	**
C^2	1.56	1	1.56	7.01	0.033,1	*
Residual	1.56	7	0.223,3			
Lack of fit	0.797,3	3	0.265,8	1.39	0.367,8	
Pure error	0.765,4	4	0.191,4			
Cor total	58.96	16				

Note: * indicates significant impact ($P < 0.05$), ** indicates an extremely significant impact ($P < 0.01$).

3.4.3 Optimization and confirmation of fermentation process results

Post-analysis and optimization with Design-Expert software version 13, the ideal conditions deduced for maximizing protein

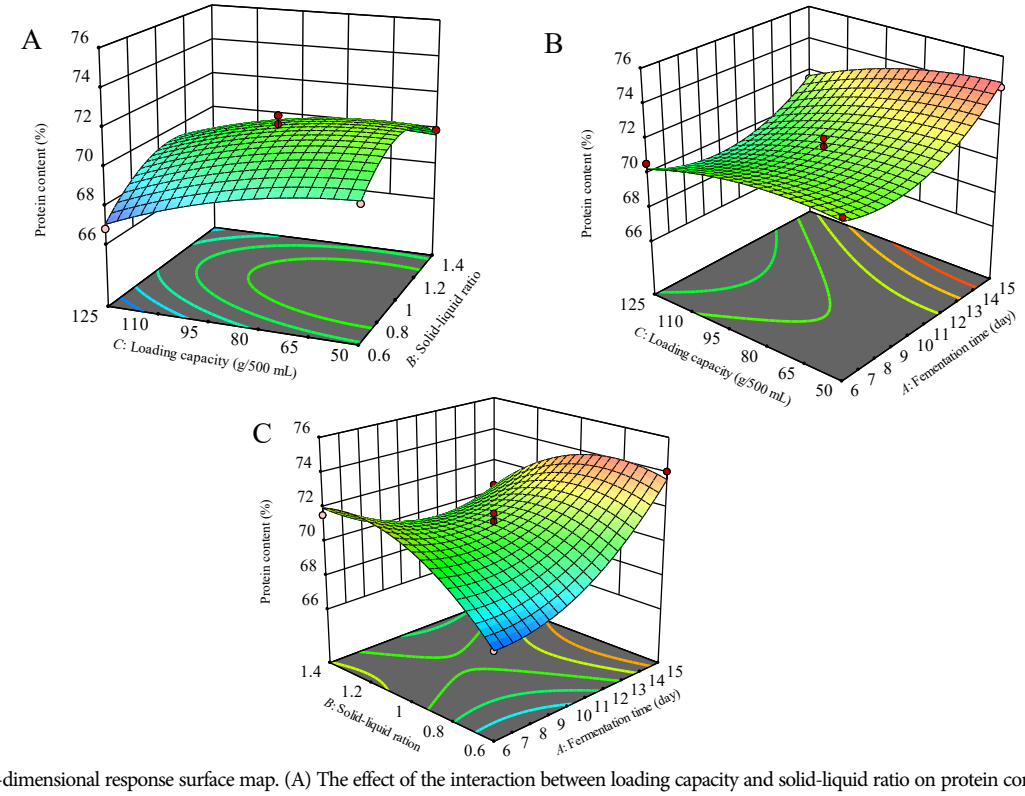

Figure 4 Three-dimensional response surface map. (A) The effect of the interaction between loading capacity and solid-liquid ratio on protein content, (B) The effect of the interaction between loading capacity and fermentation time on protein content, (C) The effect of the interaction between the solid-liquid ratio and fermentation time on protein content.

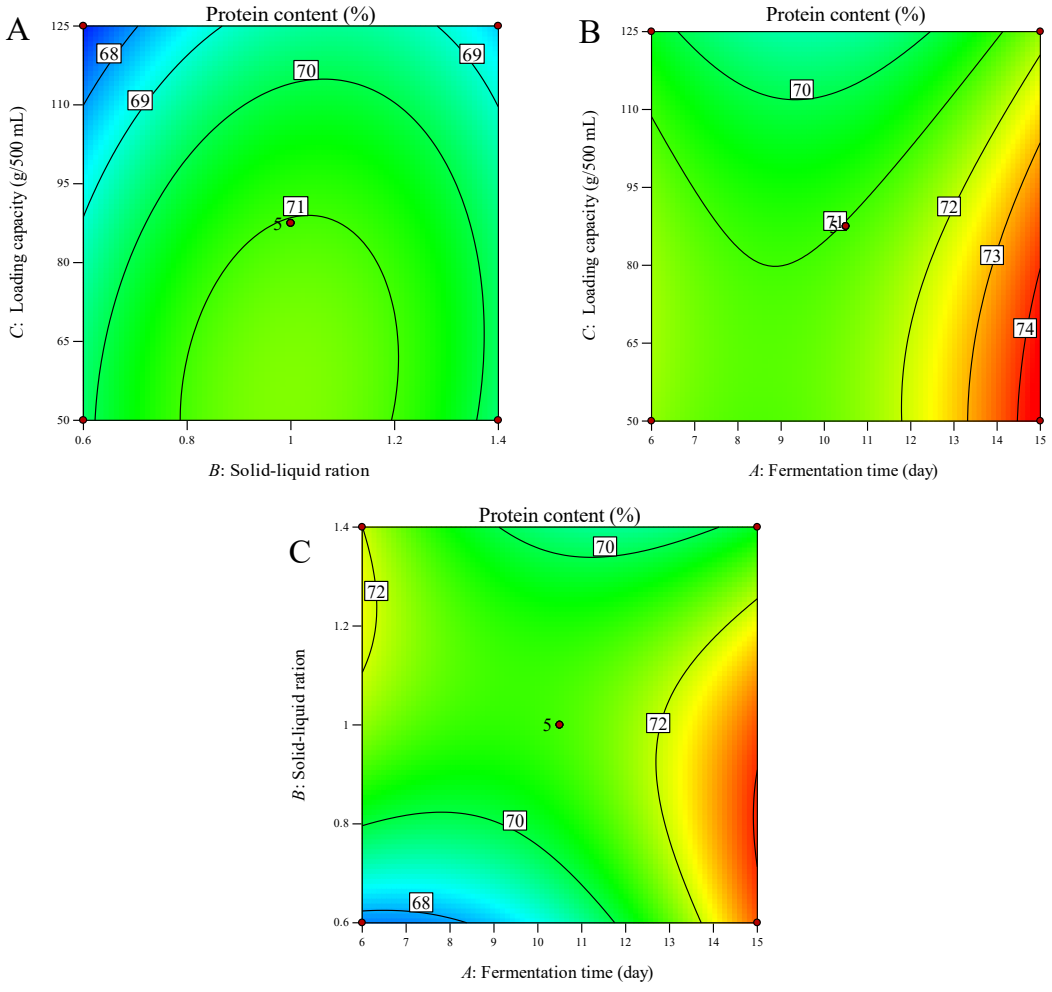

Figure 5 Contour lines. (A) The effect of the interaction between loading capacity and solid-liquid ratio on protein content, (B) The effect of the interaction between loading capacity and fermentation time on protein content, (C) The effect of the interaction between the solid-liquid ratio and fermentation time on protein content.

Table 3 Analysis of the physicochemical properties of protein residue before and after fermentation

Methord	WHC (g/g)	OHC (g/g)	EC (%)	ES (%)
Unfermented	2.46 ± 0.11[a]	1.62 ± 0.01[a]	48.80 ± 0.40[b]	15.16 ± 0.29[a]
Ferment	1.91 ± 0.27[b]	1.12 ± 0.01[b]	50.50 ± 0.30[a]	8.91 ± 0.95[b]

yield through protein residue fermentation were pegged at a fermentation span of 14.5 days, solid-liquid ratio of 1:0.8, and a loading mass of 65.59 g, predicting a protein content value of 74.30%, as shown in Table S1.

A confirmation experiment under optimized conditions was performed to assess the practicality of the response surface methodology. The parameters for this RPR/CGM SSF trial were: 14.5 days of fermentation, a solid-liquid ratio of 1:0.8, and a loading capacity of 65.59 g. Pursuing fermentation (Fig. S2) under these conditions yielded a protein content of 73.34 g per every 100 g of protein residue, amounting to a 1.29% deviation below the theoretical projection (n = 4). This outcome suggests the proposed response surface optimization conditions are viable and possess significant relevance. Currently, there exists scant research focusing on the exploitation of grain protein residue through edible fungi, which predominantly discusses its usage as a nitrogen source within liquid-state fungal cultivations[26]. So far, no literature has described the SSF of grain protein residue using edible fungi.

3.5 Analysis of amino acid content and nutritional value

Amino acid composition, as the basic component of protein, is an important attribute of protein nutritional value in human and animal diets. The amino acid content and nutritional value of different grain protein residues were shown in Table 4. The order of total amino acid content was FPR (578.66 mg/g) > SPR (526.06 mg/g) > PRBF (512.63 mg/g). Our analysis of the amino acid composition of protein residue shows that Glu has the highest content, followed by Asp and Leu, while the proportion of thioamino acids (Met + Cys) and His was the lowest. Compared with PRBF, the total amino acid concentration of SPR slightly increased, and there was no amino acid loss caused by the high-pressure sterilization process. Compared with SPR, FPR showed a significant increase in total amino acid content, which mainly led to an increase in Asp, Lys, Glu, and Phe concentrations. However, there was a slight decrease in alkaline amino acids such as Lys and Arg, as well as neutral amino acids such as Ser, which might be related to the unstable acidic conditions associated with fermentation[27]. After fermentation, the concentrations of Glu and Asp significantly increased by 19.53% and 24.27%, respectively, which might be due to their being the main amino acids in edible fungi[28].

The AAS value (Table 4) was used as an indicator to measure the protein quality in human nutrition. The results show that Lys was the first-limiting amino acid. After amino acid scoring

Table 4 Amino acid composition, EAA/TAA, AAS, SRC, EAAI, NI and PER

Nutritional indices	PRBF	SPR	FPR	WHO/FAO/UNU 2007	AAS		
					PRBF	SPR	FPR
Aspartic (mg/g)	44.27	45.95	57.10				
Serine (mg/g)	25.92	27.54	26.80				
Glutamic (mg/g)	106.28	110.72	132.34				
Glycine (mg/g)	22.38	23.17	25.46				
Alanine (mg/g)	27.05	28.91	30.33				
Proline (mg/g)	25.89	25.11	25.61				
Arginine (mg/g)	40.12	40.54	35.40				
Threonine (mg/g)	17.95	18.51	22.35	23.00	0.78	0.80	0.97
Valine (mg/g)	29.96	30.75	32.31	39.00	0.77	0.79	0.83
Methionine (mg/g)	13.75	13.29	13.48	22.00	0.63	0.60	0.61
Isoleucine (mg/g)	20.60	20.70	22.89	30.00	0.69	0.69	0.76
Leucine (mg/g)	41.33	42.30	40.90	59.00	0.70	0.72	0.69
Phenylalanine (mg/g)	26.93	27.17	31.45	30.00	0.90	0.91	1.05
Lysine (mg/g)	14.71	14.92	21.51	45.00	0.33	0.33	0.48
Cystine (mg/g)	15.58	15.95	16.38	22.00	0.71	0.73	0.74
Histidine (mg/g)	11.78	11.71	13.30	30.00	0.79	0.78	0.89
Tyrosine (mg/g)	28.15	28.84	31.05	15.00	0.94	0.96	1.04
EAA/TAA (mg/g)	0.43	0.43	0.42				
SRC	78.51	78.17	78.62				
EAAI	51.24	50.84	51.40				
NI	67.58	68.27	76.17				
PER1	16.95	17.42	16.76				
PER2	15.34	15.71	14.84				
PER3	12.31	12.20	9.44				

calculation, the amino acids in the PRBF did not meet the requirements of the WHO, FAO, and UNU standard model (>100%). The Phe and Tyr AASs of the protein residue after fermentation were greater than 100%, indicating complete protein. In addition, Thr, Val, and His all exceed 80%, indicating that the amino acid content and combination of the protein residue after fermentation could meet the needs of the human body, and the absorption and utilization rate of the human body were high.

A high EAA/TAA ratio indicates a higher content of EAAs in the protein source, which usually indicates that the protein has better quality and can better meet the amino acid needs of the human body. The EAA/TAA ratio of FPR was slightly lower than that of SPR and PRBF. It was related to a significant increase in non-essential amino acids (Glu and Asp). The higher the SRC value, the closer the composition of EAAs in the sample protein is to the model protein, indicating a higher nutritional value of the protein. After fermentation, the SRC slightly increased, indicating a more balanced proportion of EAAs. The closer the EAAI is to 100, the better the protein balance of the food. After calculation, the difference in protein residue before and after fermentation was not significant, and the EAAI value was much lower than 100, indicating that the balance of amino acids was relatively poor. NI is based on EAAI but weights the percentage of protein in food, and the weighted FPF was significantly higher than SPR and PRBF. After fermentation, the PER value of the protein residue slightly decreased, indicating a decrease in digestibility.

3.6 Analysis of physicochemical characteristics

WHC reflects the ability of proteins to retain water upon absorption. Typically, the water associated with proteins includes both free water and bound water. Proteins with an abundance of hydrophilic groups that can bind to free water molecules exhibit greater WHC. The capacity for water-holding in proteins allows high-moisture materials to flow smoothly through an extruder, aligning with the principles of high-moisture extrusion technology. Table 3 displays the WHC for protein residue pre- and post-fermentation. The WHC of the FPR fell by 22.35% compared to its unfermented counterpart. WHC is dependent on the accessibility and availability of hydrophilic amino acid side chains to interact with water[29]. The results of SSF of lupine powder using soybean *Aspergillus* and *Aspergillus flavus* reported by Olukomaiya *et al.*[30] were consistent with the above conclusion. They found that the WHC of fermented lupine powder decreased, but its swelling ability increased. The decrease in WHC might be due to a reduction in hydrophilic groups that bind to water molecules during the fermentation process. The protein hydrolysis activity caused by fungal fermentation alters the structure of proteins. As proteases hydrolyze peptide bonds, the number of polar amino acid side chains increases, resulting in higher WHC[31]. However, it is worth noting that fermentation can degrade starch and fiber. If the increase in protein was less than the decrease in starch and fiber, the WHC might decrease. Therefore, unfermented protein residue contained higher starch and fiber content, which could more effectively retain moisture.

OHC can differ due to variability in protein structure, the types of oils involved, and any emulsifiers present[18]. Proteolytic enzymes also play a role in exposing hydrophobic sites, which results in protein cleavage. These sites allow for the aggregation of proteins into clusters with microcapillaries between them, effectively trapping oil, which increases the OHC[31]. Table 3 illustrates a marked difference in OHC between protein residues before and after fermentation. The FPR's OHC decreased by 30.86% compared with the unfermented form. Gautheron *et al.*[32] reported that SSF of quinoa powder using *Rhizopus oligosporus* reduced OHC, which was consistent with the above conclusion. This might be attributed to the fact that there were fewer hydrophobic groups exposed through protein hydrolysis activity than hydrophilic groups[33].

For meat products like hamburgers and sausages, additives with high EC and ES can mitigate cooking-induced losses and shrinkage[18]. The fermentation-treated protein residue exhibited a 3.5% increase in EC but a reduction in ES. Poor solubility of Pleurotus edodes mycelium protein, which compromises foaming capabilities and ES, had been noted[34]. This decline (41.23%) in the FPR's ES might be linked to the low solubility of the edible mushroom mycelium[34].

4 Discussion

In microbial fermentation, it is necessary to comprehensively consider the effects of various culture conditions such as nitrogen source, carbon source, fermentation time, and water content on the synthesis of target products. These factors play a crucial role in determining cellular physiological characteristics and the production of specific target products. Especially the initial moisture content has a profound impact on hyphal development. Low initial moisture content cannot support the full growth of hyphae, while high moisture content is not conducive to the downward growth of hyphae, resulting in poor fermentation efficiency[35]. In addition, the thickness of the culture medium has an impact on the synthesis efficiency of the target product, as

shown in Fig. 3A. The higher the thickness, the longer the fermentation days. Therefore, these parameters must be carefully regulated during microbial fermentation experiments to enhance production efficiency. Current research on SSF of edible mushrooms primarily examines changes in nutritional composition, functional components, and antioxidant activities pre- and post-fermentation[9,36]. This study employed a high-protein substrate as the culture medium for edible mushrooms, yielding a protein-rich fermentation substrate and suggesting a novel strategy for creating artificial meat ingredients. Currently, mycelium from edible fungi is being explored in artificial meat applications across three research avenues: 1) Processing edible fungal mycelium into meat or meatball alternatives using technological methods[37], 2) Blending mushroom mycelium with traditional meat or other protein types, followed by extrusion or similar techniques to craft meat analogs[38], and 3) Utilizing active components in mycelium to enhance the quality, processing attributes, and health aspects of meat products, aiming for superior flavor and taste[39]. Hence, this study introduces an innovative methodology and direction, contributing significantly to the progress in developing artificial meat components.

Whether the amino acid pattern of protein raw materials in plant-based meat products is balanced affects the nutritional quality of plant-based meat products. The amino acid composition of grain protein is not ideal, lacking Lys and Thr. Therefore, in order to achieve a more balanced amino acid composition in plant-based meat products, functional proteins from different sources, such as fungal protein, algal protein, lupin protein, chickpea protein, etc., were commonly added[40]. Fungal protein has low allergenicity[40] and also contains trace nutrients such as vitamin B_{12}, zinc, selenium, etc. Asgar and other researchers[41] found that some fungal proteins could also regulate human blood sugar levels and help treat diabetes. In this study, after fermentation of grain protein residue, its Lys and Thr levels were increased to varying degrees, making it a highly promising source of high-quality protein.

5 Conclusion

To summarize, this study utilized response surface methodology to optimize the SSF conditions for RPR and CGM. The identified optimal conditions entailed a fermentation duration of 14.5 days, a solid-liquid ratio of 1:0.8, and a substrate loading capacity of 65.59 g. With these parameters, protein content reached 73.34 g per 100 g of dry protein residue, diverging by 1.29% from the predicted value, indicating a close agreement between experimental and theoretical data. The model derived from the study effectively optimized the SSF process for RPR and CGM. In addition, the study also examined the nutritional value and physicochemical properties of FPRs, noting a relative increase in amino acid content and emulsifying ability compared to pre-fermentation levels and evaluating their preliminary application in meat product formulations.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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Electronic Supplementary Material (ESM)

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