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Optimization of Soybean Meal Fermentation for Aqua-Feed with *Bacillus subtilis natto* Using the Response Surface Methodology

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Abstract: This study aimed to improve the nutritional value of soybean meal (SBM) by solid-state fermentation (SSF) using *Bacillus subtilis natto* (*B. s. natto*) to overcome the limitations of SBM usage in aquafeed. The response surface methodology (RSM) was employed to explore the relationships of fermentation conditions, such as temperature, time, water-substrate ratio, and layer thickness, on the degree of protein hydrolysis (DH) and the crude protein (CP) content. The optimum conditions for achieving the higher DH (15.96%) and CP (55.76%) were 43.82 °C, 62.32 h, 1.08 of water-substrate ratio, and a layer thickness of 2.02 cm. CP and DH in the fermented soybean meal (FSM) increased by 9.8% and 177.1%, respectively, and crude fiber decreased by 14.1% compared to SBM. The protein dispersibility index (PDI) decreased by 29.8%, while KOH protein solubility (KPS) was significantly increased by 17.4%. Flavonoids and total phenolic acid content in FSM were increased by 231.0% and 309.4%, respectively. Neutral protease activity (NPA) also reached a high level (1723.6 U g^{-1}). Total essential amino acids (EAA) in FSM increased by 12.2%, higher than the 10.8% increase of total non-essential amino acids (NEAA), while the total free amino acids content was 12.76 times higher than that of SBM. Major anti-nutritional factors in SBM were significantly reduced during the process, and almost all SBM protein macromolecules were decomposed. Together with the cost-effectiveness of SSF, *B. s. natto*-fermented SBM products have great potential to improve the plant composition and replace high-cost ingredients in aquafeed, contributing to food security and environmental sustainability.

Keywords: *Bacillus subtilis natto*; response surface methodology; soybean meal; solid-state fermentation; anti-nutritional factors

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1. Introduction

For over 60 years, the growth rate of the global apparent food fish consumption has exceeded that of the world population, and it is estimated that about 59% of fish available for human consumption will come from aquaculture production by 2030, up from 52% in 2018 [1]. Fishmeal has been used for most of that period as the major aquafeed,

especially for species with high nutritional needs (carnivores), due to the high quality and digestibility of its proteins, its balanced amino acid composition, and the quasi-inexistence of anti-nutritional factors (ANFs) [2–4]. However, the increasing demand for protein in the aquaculture industry has greatly affected the supply and price of fishmeal [5]. In addition, overuse of fishmeal may cause a series of environmental problems [6], and higher dependence of aquafeed on fishmeal undermines both marine biodiversity and human food security [7,8]. Therefore, the replacement of fishmeal in aquaculture is a major issue related to economic and environmental sustainability [9–11].

Widely used as one of the best alternatives to fishmeal, soybean meal (SBM) has partially or completely replaced fishmeal in animal feed [12,13] but shows imbalanced amino acid composition, high level of carbohydrate content, and potential ANFs, which may lead to adverse effects on digestion, nutrient utilization, and intestinal health [14–17]. Fish enteritis caused by SBM proteins has become one of the main challenges to sustainable aquaculture [18–20]. Proteins that are not fully digested or absorbed can be broken down into ammonia by *E. coli, Aspergillus, Salmonella*, and other microorganisms in the digestive tract or excreted in feces, resulting in protein waste and environmental pollution [21].

Current studies have shown that microbial fermentation is an effective method to reduce ANFs from SBM [22]. Fermentation improves the digestibility of amino acids and phosphorus in SBM [23], and the fermentation products contain beneficial growth factors, such as proteases, probiotics, organic acids, and small molecular peptides [24,25]. It can improve the nutritional performance of SBM, thus expanding the scope of its usage [26,27].

Solid-state fermentation (SSF) has a long history as a traditional method of food production using diverse organisms, and SSF has been reported to produce more metabolites, such as enzymes and antibiotics, than submerged fermentation [28,29]. SSF comes with many advantages. First, the culture conditions used in SSF are relatively extensive, and the culture medium only needs relatively simple pre-treatment; secondly, the capital investment in industrial production is relatively small, especially in the early stage of industrial production, which is conducive to large-scale production; in addition, the product performance with SSF is better, and the environmental pollution in industrial production is less, which is more conducive to the biological cycle [30–32]. SSF with *Bacillus subtilis* is used to produce traditional fermented soy food, Natto, in Japan. Natto contains functional compound enzymes, bioactive peptides, natto kinase, and γ-polyglutamic acid, as well as vitamins, soy-derived isoflavones, linoleic acid, dietary fiber, and many minerals [33]. *Bacillus subtilis natto* (*B. s. natto*) is the probiotic spore-forming bacterium in Natto, a kind of strain with a long history of human consumption known for its definite effect on human health and food safety [34,35]. *B. s. natto* has attracted much attention in the animal nutrition and feed industry due to its enzymatic, antioxidative, immunomodulatory, anti-inflammatory, and antimicrobial activities [36]. However, there are still only a few studies of the use of *B. s. natto* fermented soybean meal (FSM) in aquatic feed.

In the present study, SSF technology was used for the fermentation of SBM by *B. s. natto*. To improve the protein content and digestibility of SBM, the response surface methodology (RSM) based on Central Composite Design was used to evaluate and optimize the effects of temperature, fermentation time, the ratio of water, and bacterial inoculation quantity during the fermentation, targeting the degree of protein hydrolysis (DH) and the crude protein (CP). After completing the optimization of the fermentation factors, the nutrient composition of the SBM before and after fermentation was examined. The effects of fermentation on protein dispersibility index (PDI), KOH protein solubility (KPS), and active antioxidant substances were compared; the neutral protease activity (NPA) secreted by *B. s. natto* was also tracked over the SSF process. Changes to the amino acids' profile were analyzed. Various soybean anti-nutritional factors were also determined, and the examination of the degradation of soybean antigenic proteins and the distribution of peptides by SDS-PAGE was carried out.

2. Materials and Methods

2.1. Plant Materials

Defatted SBM containing 50.72 ± 0.30% protein (dry matter basis) was obtained from J-OIL MILLS, Inc. (Tokyo, Japan).

2.2. Starter Culture Preparation

B. s. natto powder (product name: np1) was obtained from Yuzo Takahashi Laboratory, Co., Ltd. (Kaminoyama, Japan).

For inoculum preparation, *B. s. natto* was activated in Luria–Bertani (LB) liquid medium (pH 7.5) [37] for 24 h at 37 °C with shaking. The cells were adjusted to 10^9 colony forming units (CFU)/mL with sterilized physiological saline solution and used as inoculum for SSF.

2.3. Solid State Fermentation and Single Factor Tests

SSF was carried out with 100 g of crushed SBM in an aluminum plate after autoclaving (121 °C/20 min). Based on the basic SSF conditions (Table 1.), single-factor conventional optimizations were carried out with the inoculation quantities (10^7, 10^8,10^9,10^{10},10^{11} CFU/kg) of *B. s. natto* in fermentation substrate with the following conditions; culture temperature (31, 34, 37, 40, 43, 46 °C), fermentation time (24, 36, 48, 60, 72, 84 h), water–material ratio (0.4, 0.7, 1.0, 1.3, 1.6, 1.9 *v*/*w*), and SBM layer thickness (1.0, 1.5, 2.0, 3.0, 4.0, 5.0 cm). The selection of fermentation conditions using RSM was based on the DH.

Table 1. Basic solid-state fermentation (SSF) conditions.

Fermentation Strain	Inoculation Quantity (log CFU/kg)	Temperature (°C)	Time (h)	Water–Material Ratio	Layer Thickness (cm)	Initial pH
B. s. natto	10.0	40.0	48.0	1.0	2.0	7.5

2.4. Optimization of Fermentation Process by RSM

Two responses were selected to study the optimized conditions for fermentation. Using DH and CP as response target variables, RSM was applied to determine the optimum levels of four significant variables: culture temperature (A), fermentation time (B), water–material ratio (C), and layer thickness (D) [38]. A 2^4 factorial central composite design (CCD) with five coded levels ($-\alpha$, -1, 0, +1, $+\alpha$ ($\alpha = 2$)) and six replicates at the central point used to estimate the pure error was used to describe the nature of the response surface in the optimum region [39]. The minimum and maximum ranges of variables investigated are listed in Tables 2 and 3, listing the whole design consisting of 30 experimental points, and the experiment was carried out at random [40].

Table 2. Ranges of the four independent variable variations used in response surface methodology (RSM).

Code	Independent Variables	Levels				
		$-\alpha$	−1	0	+1	$+\alpha$
A	Temperature (°C)	31.00	36.00	41.00	46.00	51.00
B	Time (h)	54.00	60.00	66.00	72.00	78.00
C	Water–material ratio	0.90	1.00	1.10	1.20	1.30
D	Layer thickness (cm)	1.60	1.80	2.00	2.20	2.40

Code A: fermentation temperature; code B: fermentation time; code C: water–material ratio; code D: layer thickness.

Table 3. Experimental designs used in RSM studies for optimization of fermented soybean meal (FSM).

Runs	A	B	C	D	Response 1: CP (%)	Response 2: DH (%)
1	36.00	72.00	1.20	1.80	54.86	15.53
2	36.00	60.00	1.00	1.80	55.24	15.73
3	41.00	66.00	1.10	2.00	55.59	15.94
4	41.00	66.00	1.10	2.40	53.64	15.56
5	41.00	66.00	1.10	2.00	55.64	15.93
6	46.00	60.00	1.20	2.20	55.49	15.86
7	46.00	72.00	1.20	1.80	54.36	15.53
8	46.00	72.00	1.20	2.20	54.91	15.87
9	51.00	66.00	1.10	2.00	55.15	15.89
10	41.00	54.00	1.10	2.00	55.53	15.76
11	36.00	72.00	1.20	2.20	54.81	15.67
12	41.00	66.00	1.10	1.60	53.99	15.36
13	31.00	66.00	1.10	2.00	55.05	15.62
14	41.00	66.00	0.90	2.00	55.16	15.87
15	41.00	66.00	1.10	2.00	55.83	15.92
16	46.00	60.00	1.00	2.20	55.03	15.85
17	36.00	72.00	1.00	2.20	54.25	15.65
18	46.00	72.00	1.00	2.20	54.53	15.85
19	46.00	60.00	1.00	1.80	55.34	15.80
20	36.00	72.00	1.00	1.80	55.15	15.71
21	41.00	66.00	1.10	2.00	55.75	15.94
22	36.00	60.00	1.00	2.20	54.40	15.56
23	41.00	78.00	1.10	2.00	54.87	15.78
24	46.00	60.00	1.20	1.80	54.87	15.59
25	46.00	72.00	1.00	1.80	54.84	15.72
26	41.00	66.00	1.10	2.00	55.75	15.93
27	36.00	60.00	1.20	2.20	55.11	15.56
28	41.00	66.00	1.10	2.00	55.65	15.95
29	41.00	66.00	1.30	2.00	55.30	15.68
30	36.00	60.00	1.20	1.80	55.01	15.50

Code A: fermentation temperature; code B: fermentation time; code C: water–material ratio; code D: layer thickness. CP: crude protein; DH: degree of protein hydrolysis.

The experimental results of the CCD were fit with a second-order polynomial equation by multiple regression techniques.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j=2}^{4} \beta_{ij} X_i X_j + e_i \quad (1)$$

where Y is the predicted response, β_0 is the model intercept coefficient, β_i, β_{jj}, and β_{ij} are regression coefficients of the linear, quadratic, and interactive terms, respectively, and e_i is the error [41,42].

To validate the modeling results, three independent iterations of the experiment were carried out using optimized conditions representing the maximum points of DH and CP.

2.5. Samples

Samples were collected daily and stored at −20 °C pending the determination of their respective nutritive compositions. Before analysis, all SBMs and FBMs were ground in a rotary mill with a 0.2 mm mesh sieve.

2.6. Degree of Protein Hydrolysis Analysis

The DH was defined as the percentage of peptide bonds cleaved during a reaction and calculated from the ratio of free amino groups to the total number of peptide bonds in soluble factions [43]. DH was determined using the o-phthaldialdehyde (OPA) assay

method, as described by Nielsen et al. [44]. The absorbance was measured at 340 nm with a spectrophotometer (HACH, DR/4000U) and using deionized water as the control. The DH was calculated using the formula below.

$$\mathrm{DH}(\%) = \frac{\mathrm{NH_2(\ sample\ )}}{\mathrm{NH_2\ (acid)}} \times 100\% \quad (2)$$

where NH_2 (sample) is the concentration of free amino groups of the samples, NH_2 (acid) is the total amount of free amino groups in the samples after completely hydrolyzed in 6 M HCl, 110 °C for 24 h [45].

2.7. Nutritional Analysis

The analysis of the nutritional profile was performed according to the standard procedures of AOAC for the determination of the dry matter, CP, crude lipid, and fiber [46,47]. PDI, as an indicator of overprocessing of SBM, was determined using the method of Căpriţă et al. [48]. The KPS was determined according to the procedure of Araba and Dale [49]. The total phenol content of the samples was determined using Folin–Ciocalteau's reagent with gallic acid as a standard phenolic compound [50]. Total flavonoids were analyzed according to Paolo et al. [51]. The NPA of FSM was determined according to the spectrophotometric method [52]. The obtained supernatant was designated enzyme extract and held at 4 °C for further use. Total amino acids and free amino acids were analyzed using high-performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) as described previously by Teshima et al. [53]. TAA and FAA samples were prepared according to Kader et al. [54]. All analyses were performed in triplicate.

2.8. ANFs Assays of SBM and FSM

Trypsin inhibitors were measured in this study using American Oil Chemists' Society (AOCS) Official Method Ba 12–75 [55]. The concentration of β-conglycinin was measured using an ELISA procedure [56]. Glycinin was also measured using the Glycinin ELISA kit (Wuhan Unibiotest Co., Ltd., Wuhan, China). Lectin content was assayed according to the method of Pusztai and Grant using an enzyme-linked immunosorbent assay [57]. Raffinose and stachyose were extracted from bean flour using the method from Zacharie and Ronald [58], and the contents were quantified by using an HPLC system described previously [59] with an HPLC column Inertsil® NH2 (4.6 × 250 mm, 5 µm; GL Sciences Inc., Tokyo, Japan). Phytic acid content was determined spectrophotometrically based on the method from Reichwald and Hatzack [60]. All experiments were replicated three times.

2.9. SDS-Polyacrylamide Gel Electrophoresis (PAGE) for Protein Separation

Proteins in SBM and FSM were extracted by the method reported by Hong et al. [25]. Soluble proteins were analyzed by SDS-PAGE in the Tris-glycine method using a mini PAGE chamber AE-6530 and an AE-8750 electrophoresis system (ATTO, Tokyo, Japan) with e-PAGEL® HR precast gels (ATTO, Tokyo, Japan). A total of 10 µg of extracted protein was loaded for each well, and the samples were separated at 20 mA for 90 min. Precision Plus Protein™ Dual Color Standards (Bio-Rad Cat# 161-0374; CA, USA) were used as molecular mass standards. After electrophoresis, the gels were stained for 40 min with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid and destained overnight in a solution made up of 40% methanol and 10% acetic acid [61].

2.10. Statistical Analysis

RSM was performed using the "rsm" package in R 3.6.1 [62]. Statistical analysis of the data from the CCD model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equations was judged statistically by the coefficient of determination R^2, and its statistical significance was determined by the F-test.

One-sample *t*-test for comparison between predicted and experimental responses at the optimum condition was performed using package "stats" in R. *t*-tests for all other comparison experiments were performed using package "stats" in R.

Principal Component Analysis plots were produced in R using the "FactoMineR" package and the "ggplot2" package to visually present the selected significant attributes [63].

All experiments were replicated three times unless stated otherwise. Data were presented as mean values ± standard deviation (SD).

3. Results and Discussion

3.1. Single Factor Tests

Single-factor analysis was performed to establish the optimum DH (%) with a fermentation condition using the broken stick pairwise regression model. Considering the single factor in the fermentation process, the following optimum conditions were achieved for the establishment of the interactive effects, which could be useful when the target is only a single factor to maximize DH (%) and could be used to pilot optimum fermentation conditions using other related microbial species.

3.1.1. Effect of Inoculation Quantity of *B. s. natto* on the DH

As the initial *B. s. natto* inoculum increased (from 10^7 to 10^{10} CFU/kg), the DH presents an increasing trend, as shown in Figure 1a. With the inoculum levels exceeding 10^{10} CFU/kg, this increase does not continue. This shows that in the inoculation quantity of 10^{10} CFU per kg, fermentation substrates did not restrict the fermentation kinetics at the initial stage. Therefore, in this work, 10^{10} CFU/kg *B. s. natto* was selected as the initial optimal amount of inoculum for the following RSM experiments.

3.1.2. Effect of Fermentation Temperature on the DH

Previous studies have confirmed that temperature affects not only the growth of microorganisms but also the activity of enzymes [64]. As SSF is used for this experiment, it was particularly important to choose a suitable fermentation temperature. Based on the optimum fermentation temperature of *B. s. natto* [37], the starting point was set at 28 °C, followed by a subsequent increase of 5 °C at each experimental time point until 53 °C is reached. As shown in Figure 1b, the degree of hydrolysis of the fermented protein increases with increasing temperature from 28 to 43 °C, indicating that the activity and number of *B. s. natto* increased with the gradual increase of temperature, which led to the continuous increase of fermentation rate. However, the DH tends to decrease from 43 °C, which may be due to the fact that the fermentation temperature exceeds the optimum temperature for the growth or metabolism of *B. s. natto*, slowing down the fermentation activity. According to the broken-line analysis, 41 °C was selected as the 0 level of temperature factor for RSM.

3.1.3. Effect of Fermentation Time on the DH

As shown in Figure 1c, fermentation time has a positive effect on protein hydrolysis within 60 h. However, with the extension of fermentation time, the number of fermentation by-products increases, whereas the quality of fermentation products decreases, reflecting the growth rule of microorganisms, as *B. s. natto* gradually die out in the process. A time of 66 h was selected as the 0 level of time factor for RSM through curve-fitting analysis.

3.1.4. Effect of Water–Material Ratio on the DH

In the SBM fermentation experiment, the water content of the whole fermentation system is very important, as too high water content affects the transfer of oxygen and limits the growth of aerobic bacteria. Low water content cannot meet the water requirement for strain fermentation and inhibits the growth of bacteria. The experimental results (Figure 1d) showed that the best fermentation effect is achieved when the water–material ratio is 1.1. This was chosen as the 0 level for RSM.

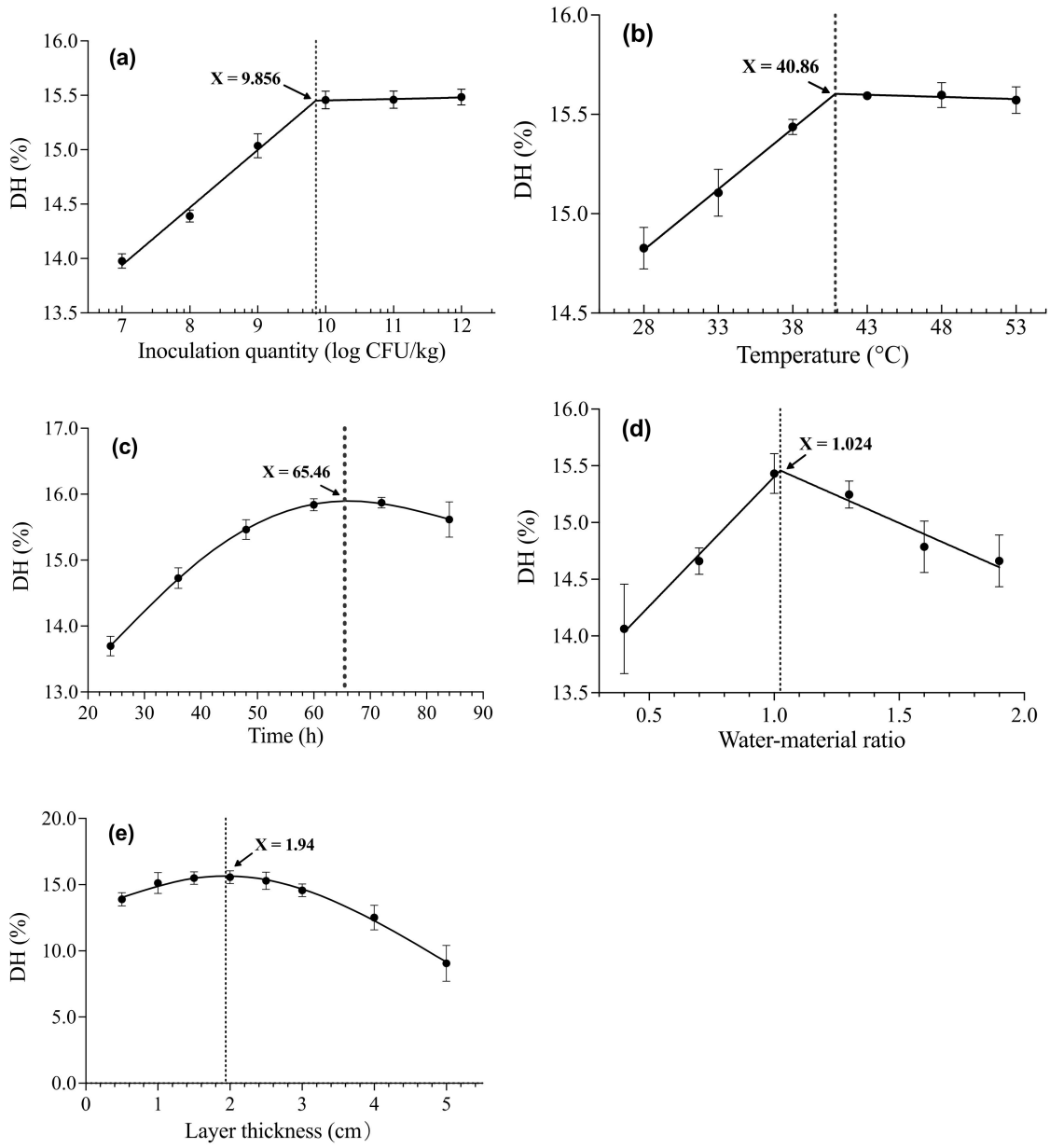

Figure 1. Single-factor test results of (**a**) inoculation quantity, (**b**) temperature, (**d**) water–material ratio by broken-line analysis and (**c**) fermentation time, and (**e**) soybean meal (SBM) layer thickness by curve fitting analysis. The value of the horizontal axis corresponding to the maximum DH value is the optimal state of the fermentation factor.

3.1.5. Effect of SBM Layer Thickness on the DH

The experimental results by curve-fitting analysis show that the DH reached the maximum when the layer thickness was 2.0 cm (Figure 1e). This is because the solid matrix is porous and is affected by the low thermal conductivity as it grows. The heat generated by microbial growth and metabolism cannot be transferred to the environment in time, resulting in the increase of matrix temperature and affecting the further growth and metabolism of microorganisms.

3.2. Further Optimization Using CCD

3.2.1. Variance and Regression Analysis of CCD Design

CCD was conducted to locate the true optimum culture temperature, fermentation time, water–material ratio, and SBM layer thickness for better DH and CP. The levels of the

variables for the CCD experiments were selected according to the results of the single factor tests. The design matrix and the corresponding experimental data are given in Table 3. DH and CP data were performed via the "rsm" package in R and fit with second-order polynomial equations. ANOVA results for the full quadratic model of CCD design on the response of DH and CP were shown in Tables 4 and 5, respectively. In order to simplify the model, the non-significant terms ($p > 0.05$) in the models were reduced. The regression analysis results of CCD are also summarized in Tables 4 and 5.

Table 4. Variance and regression analysis of central composite design (CCD) on the response of degree of protein hydrolysis (DH).

ANOVA for Quadratic Model						ANOVA for Reduced Quadratic Model					
Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	0.7664	14	0.0547	972.72	<0.0001	Model	0.7664	13	0.059	1111.15	<0.0001
A	0.1185	1	0.1185	2106.2	<0.0001	A	0.1185	1	0.1185	2234.09	<0.0001
B	0.0005	1	0.0005	8.65	0.0101	B	0.0005	1	0.0005	9.18	0.008
C	0.0534	1	0.0534	948.5	<0.0001	C	0.0534	1	0.0534	1006.1	<0.0001
D	0.053	1	0.053	942.48	<0.0001	D	0.053	1	0.053	999.71	<0.0001
AB	0.0073	1	0.0073	129.66	<0.0001	AB	0.0073	1	0.0073	137.53	<0.0001
AC	4.73×10^{-6}	1	4.73×10^{-6}	0.0841	0.7758						
AD	0.0417	1	0.0417	741.42	<0.0001	AD	0.0417	1	0.0417	786.44	<0.0001
BC	0.0009	1	0.0009	16.83	0.0009	BC	0.0009	1	0.0009	17.85	0.0006
BD	0.0072	1	0.0072	128.3	<0.0001	BD	0.0072	1	0.0072	136.09	<0.0001
CD	0.0452	1	0.0452	802.89	<0.0001	CD	0.0452	1	0.0452	851.65	<0.0001
A^2	0.057	1	0.057	1013.04	<0.0001	A^2	0.057	1	0.057	1074.56	<0.0001
B^2	0.05	1	0.05	887.75	<0.0001	B^2	0.05	1	0.05	941.66	<0.0001
C^2	0.0479	1	0.0479	850.7	<0.0001	C^2	0.0479	1	0.0479	902.36	<0.0001
D^2	0.392	1	0.392	6964.4	<0.0001	D^2	0.392	1	0.392	7387.3	<0.0001
Residual	0.0008	15	0.0001			Residual	0.0008	16	0.0001		
Lack of Fit	0.0004	10	0	0.478	0.85	Lack of Fit	0.0004	11	0	0.4395	0.8811
Pure Error	0.0004	5	0.0001			Pure Error	0.0004	5	0.0001		
Cor Total	0.7673	29				Cor Total	0.7673	29			
Credibility analysis of the regression equations for Quadratic model						Credibility analysis of the regression equations for Reduced Quadratic model					
Std. Dev.	0.0075		R^2	0.9989		Std. Dev.	0.0073		R^2	0.9989	
Mean	15.74		Adjusted R^2	0.9979		Mean	15.74		Adjusted R^2	0.998	
C.V. %	0.0477		Predicted R^2	0.9961		C.V. %	0.0463		Predicted R^2	0.9967	

Code A: fermentation temperature; code B: fermentation time; code C: water–material ratio; code D: layer thickness.

The goodness of fit of the model based on RSM can be checked by the coefficient of determination (R^2), which provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions [65]. In this case, in the model of DH response, the coefficient of determination (adjusted $R^2 = 0.998$) indicated that 99.8% of the variability in the response could be explained by the model. At the same time, the "lack of fit" was non-significant relative to the pure error ($p = 0.8811$). The model term, the coefficients of the linear term (A, B, C, and D), the quadratic term (A^2, B^2, C^2, and D^2), and the interaction term (AB, AD, BC, BD, and CD), with *p*-values of less than 0.05, demonstrated that they were significant for the DH.

Table 5. Variance and regression analysis of CCD on the response of crude protein (CP).

ANOVA for Quadratic Model						ANOVA for Reduced Quadratic Model					
Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	8.29	14	0.5924	148.94	<0.0001	Model	8.28	12	0.6903	168.99	<0.0001
A	0.0214	1	0.0214	5.39	0.0348	A	0.0214	1	0.0214	5.24	0.0351
B	0.706	1	0.706	177.52	<0.0001	B	0.706	1	0.706	172.85	<0.0001
C	0.0358	1	0.0358	9	0.009	C	0.0358	1	0.0358	8.76	0.0088
D	0.1415	1	0.1415	35.58	<0.0001	D	0.1415	1	0.1415	34.64	<0.0001
AB	0.1222	1	0.1222	30.71	<0.0001	AB	0.1222	1	0.1222	29.91	<0.0001
AC	0.0469	1	0.0469	11.8	0.0037	AC	0.0469	1	0.0469	11.49	0.0035
AD	0.3134	1	0.3134	78.81	<0.0001	AD	0.3134	1	0.3134	76.74	<0.0001
BC	0.0054	1	0.0054	1.35	0.2633						
BD	0.0044	1	0.0044	1.11	0.3091						
CD	0.8085	1	0.8085	203.28	<0.0001	CD	0.8085	1	0.8085	197.93	<0.0001
A^2	0.5457	1	0.5457	137.22	<0.0001	A^2	0.5457	1	0.5457	133.61	<0.0001
B^2	0.3734	1	0.3734	93.89	<0.0001	B^2	0.3734	1	0.3734	91.42	<0.0001
C^2	0.3226	1	0.3226	81.12	<0.0001	C^2	0.3226	1	0.3226	78.99	<0.0001
D^2	5.86	1	5.86	1472.95	<0.0001	D^2	5.86	1	5.86	1434.18	<0.0001
Residual	0.0597	15	0.004			Residual	0.0694	17	0.0041		
Lack of Fit	0.0198	10	0.002	0.2477	0.9711	Lack of Fit	0.0295	12	0.0025	0.3086	0.9558
Pure Error	0.0399	5	0.008			Pure Error	0.0399	5	0.008		
Cor Total	8.35	29				Cor Total	8.35	29			
Credibility analysis of the regression equations for Quadratic model						Credibility analysis of the regression equations for Reduced Quadratic model					
Std. Dev.	0.0631		R^2	0.9929		Std. Dev.	0.0639		R^2	0.9917	
Mean	55.04		Adjusted R^2	0.9862		Mean	55.04		Adjusted R^2	0.9858	
C.V. %	0.1146		Predicted R^2	0.9795		C.V. %	0.1161		Predicted R^2	0.9781	

Code A: fermentation temperature; code B: fermentation time; code C: water–material ratio; code D: layer thickness.

In the model of CP response, the coefficient of determination R^2 = 0.9917, which implied that the CP content was attributed to the given independent variables. The value of the adjusted determination coefficient (adjusted R^2 = 0.9858) was also high to indicate a high significance of the model. The model also showed a statistically insignificant lack of fit, as the p = 0.9558. These measures indicated that the accuracy and general ability of the polynomial model were good and that analysis of the response trends using the model was reasonable. The model term, the coefficients of the linear term (A, B, C, and D), the quadratic term (A^2, B^2, C^2, and D^2), and the interaction term (AB, AC, AD, and CD), with p-values of less than 0.05, demonstrated that they were significant for the CP.

The polynomial models for the DH (Y_{DH}) and CP (Y_{CP}) were regressed by only considering the significant terms and were shown as below.

$$Y_{DH} = 4.51035 + 0.159515X_A + 0.154623X_B + 5.21652X_C + 0.013533X_D - 0.000712X_AX_B + 0.025534X_AX_D + 0.012823X_BX_C + 0.008852X_BX_D + 1.32859X_CX_D - 0.001824X_A^2 - 0.001186X_B^2 - 4.17802X_C^2 - 0.747142X_D^2 \quad (3)$$

$$Y_{CP} = 14.35734 + 0.640053X_A + 0.51865X_B + 17.44795X_C + 2.3107X_D - 0.002912X_AX_B - 0.108325X_AX_C + 0.069981X_AX_D + 5.61969X_CX_D - 0.005642X_A^2 - 0.003241X_B^2 - 10.84542X_C^2 - 2.88839X_D^2 \quad (4)$$

The typical analysis of the model was carried out by using the R3.6.1 package, and the optimum fermentation conditions for the maximum DH and CP of FSM were obtained as follows (Figure 2): fermentation temperature 43.82 °C, fermentation time 62.32 h, water–material ratio 1.08, and SBM layer thickness 2.02 cm. The theoretical maximum of DH was 15.96% and CP was 55.76%.

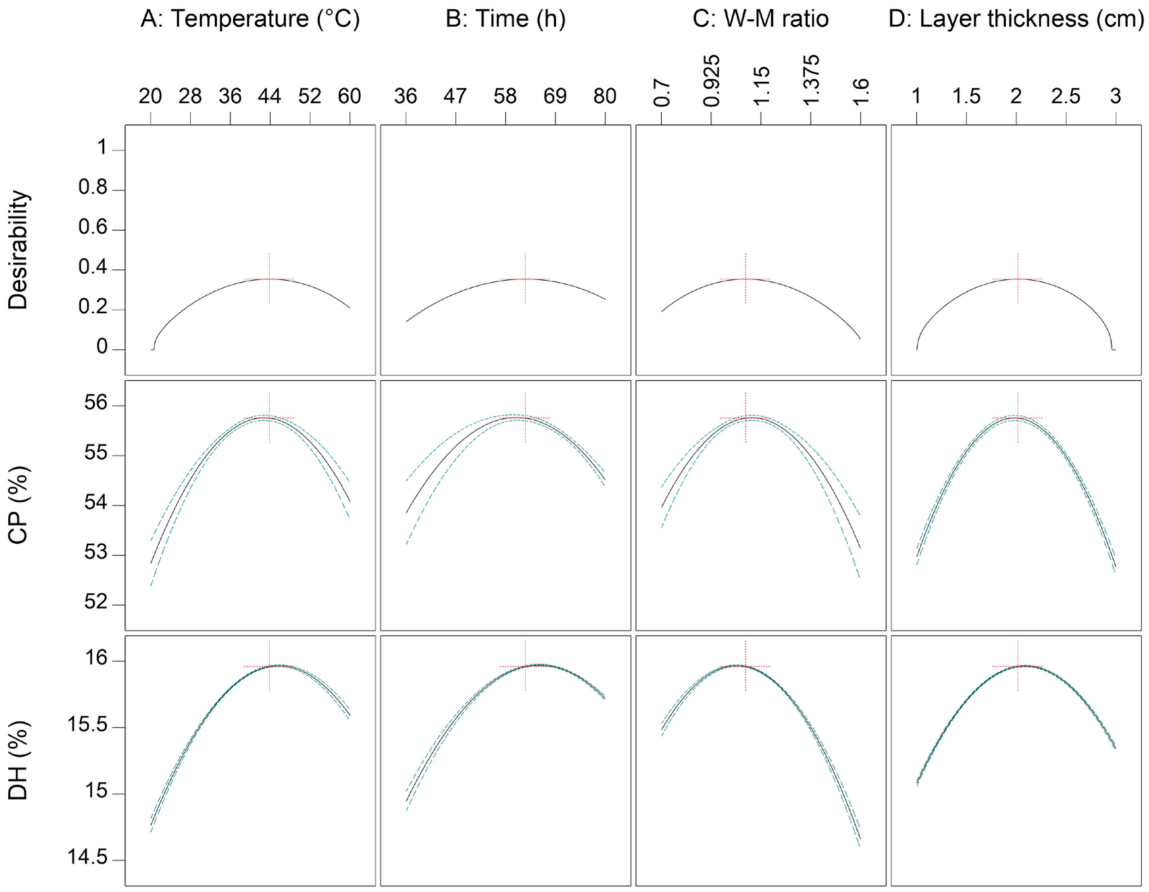

Figure 2. The optimization results of RSM with all responses. Factor A: Temperature = 43.82; B: Time = 62.32; C: Water–material ratio = 1.08; D: Layer thickness = 2.02. Responses DH = 15.96; CP = 55.76; Overall Desirability = 0.35.

3.2.2. Response Surface Interaction Analysis

In order to intuitively study the interaction between the four factors and the response, models were plotted as a 3D surface representing the responses (DH and CP) as a function of two factors within the tested processing parameters. The shape of the response surface graph can reflect the interaction between the two factors and the steep slope of the figure, indicating that the interaction between the two factors is significant.

In Figures 3a and 4a, the 3D response surface plots and contour plots were developed for the DH and CP with temperature (A) and time (B), with the other factors' optimal values. The interactive effects between temperature and time revealed significant effects. Increasing the initial temperature can increase the initial efficiency of fermentation, but with the progress of fermentation, the heat production of fermentation accumulates in the SBM medium, which is not conducive to fermentation when it exceeds the optimum temperature of microorganisms. If the initial fermentation temperature is too low (such as room temperature), even if the fermentation time is prolonged, a good fermentation effect cannot be achieved. Therefore, the suitable combination of temperature and time is very important, which can get the best fermentation effect and have a better economy in terms of energy-saving and production speed. Maximum DH and CP were achieved when fermentation temperature and time were 43.8 °C and 62.3 h, respectively.

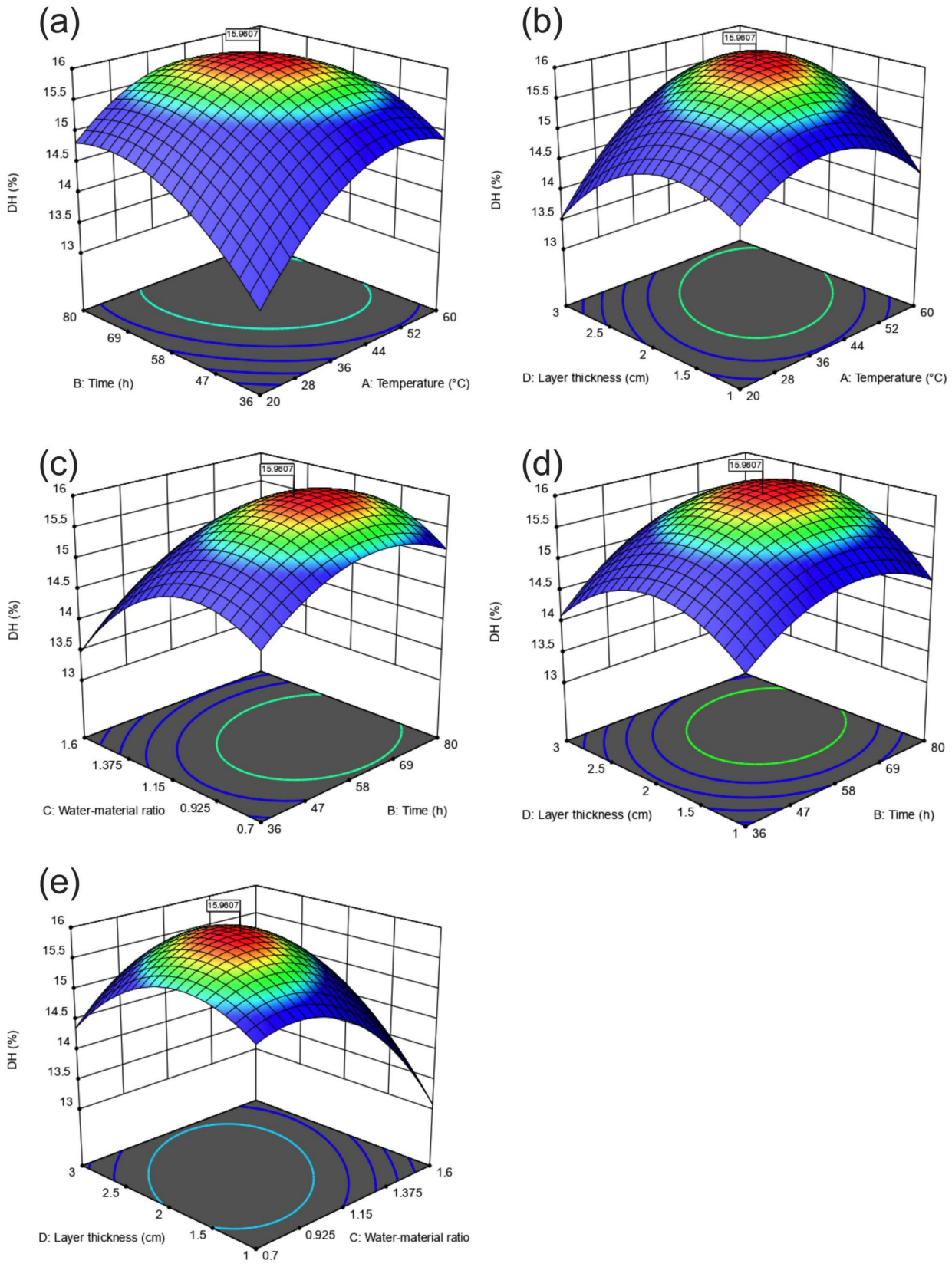

Figure 3. Optimization of the DH using CCD. Interaction of time and temperature (**a**); interaction of temperature and water–material ratio (**b**); interaction of time and water–material ratio (**c**); interaction of time and layer thickness (**d**); interaction of water–material ratio and layer thickness (**e**). The green layer of the surface plot represents the optimum condition.

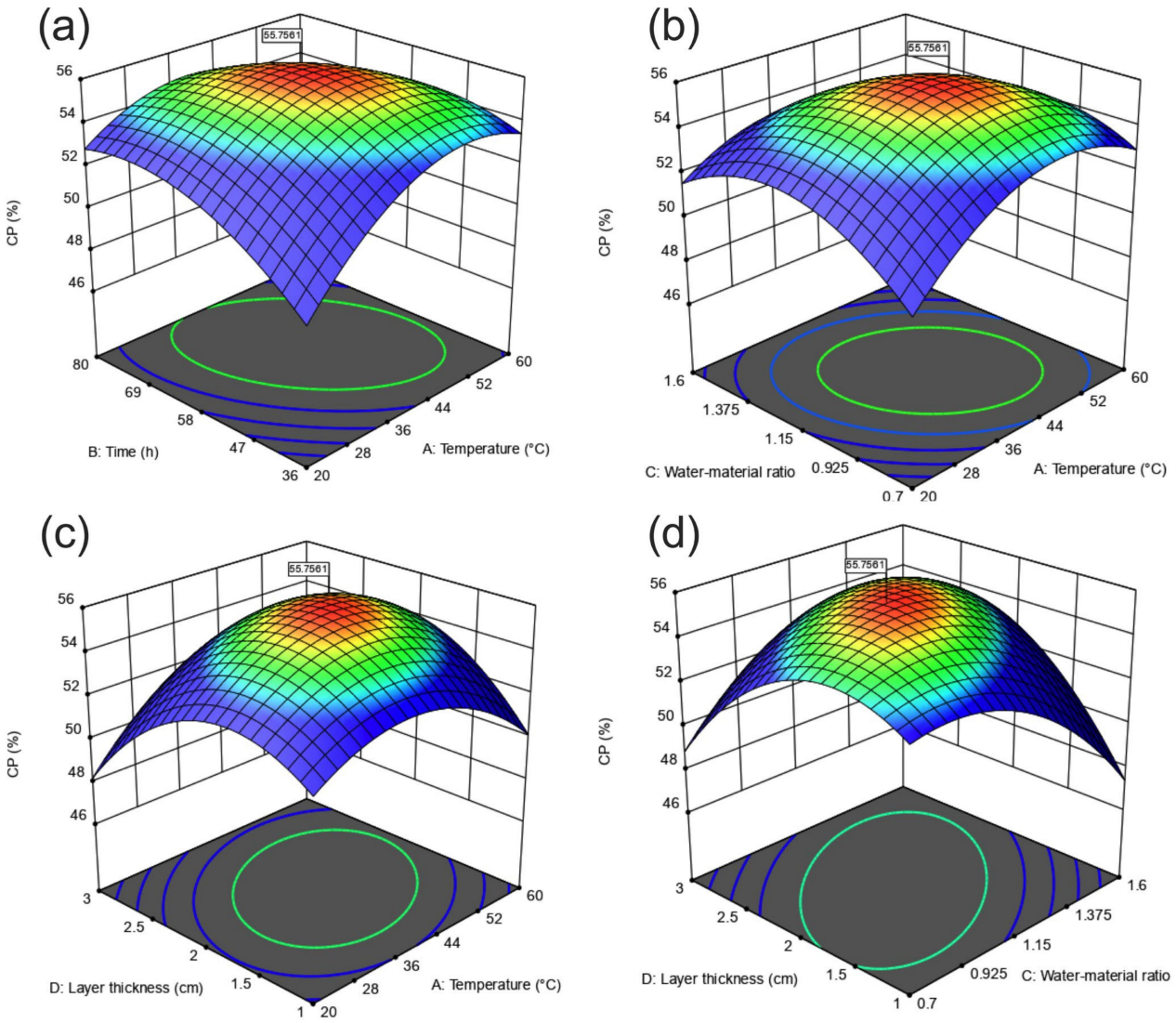

Figure 4. Optimization of CP using CCD. Interaction of time and temperature (**a**); interaction of temperature and water–material ratio (**b**); interaction of temperature and layer thickness (**c**); interaction of water–material ratio and layer thickness (**d**). The green layer of the surface plot represents the optimum condition.

Figures 3b and 4c show 3D response surface and contour plots at temperature (A) and SBM layer thickness (D), as well as at the optimal time and water–material ratio values. As can be seen from the figures, when the temperature is constant, the DH and CP content of FSM reaches the maximum when the layer thickness is about 2 cm. This is due to the porous structure of the solid matrix; when the material layer is very thin, the rapid evaporation of the water cannot meet the optimal water content of fermentation, and it is not conducive to maintaining the optimal bacterial growth temperature. If the substrate is too thick, the heat produced by microbial fermentation cannot be transferred to the environment in time, resulting in the increase of substrate temperature and affecting the fermentation efficiency of microorganisms. The elliptical nature of the contour plot in DH and CP indicates the interaction of fermentation temperature and layer thickness is considerable. The thickness of the material layer affects the fermentation temperature, and both the initial temperature and the layer thickness have a common effect on the water content in the SBM layer.

Figure 3c illustrates the interaction of fermentation time and water–material ratio on DH. The effect of fermentation time on the DH is as a quadratic curve, and the DH of FSM reaches the maximum when the ratio of water to material is about 1.08. This

is because, with the increase of water content, the water activity on the surface of SBM particles increases, which is beneficial to the growth of microorganisms; however, if the water content is too high, the pores between SBM particles will decrease, which reduces the fluidity of air on the surface of solid medium particles, resulting in the slow growth of microorganisms. Elabona et al. [66] confirmed this view through experiments and found that the endoglucanase activity produced by Aspergillus Niger decreased with the increase of water content when the water content of the SBM matrix was high. Although the interaction between water–material ratio and time is obvious for DH, it is not significant for the model of CP. It shows that the interaction between water–material ratio and time has a greater effect on the activity of protein hydrolase due to the fact that DH is mainly affected by protease decomposition, while the increase of CP content is mainly caused by the concentration of the loss of H_2O and CO_2 after the decomposition of carbohydrate and lipid substances.

Figure 3d shows that DH increased with the increase of layer thickness and fermentation time up to a critical point. Their interaction affects the efficiency of protease hydrolysis through the change of the relative concentration of substrate and heat transfer in the process of fermentation. Similarly, there was no significant interaction on CP content.

Figures 3e and 4d illustrate the interaction of layer thickness and water–material ratio on DH and CP, respectively. The 3D response surface plots show that the optimal response range of the two factors is in the shape of a ridge. As seen on the plot, the very steep slope indicated that the degree of interaction between the two factors is very strong. This phenomenon is caused by the evaporation of water in the substrate. In SSF, the growth and metabolism of microorganisms occur on the surface of the matrix particles, which is between the air (gas phase) and the matrix particles (solid phase). The water activity on the surface of the matrix is very important. The thicker the material layer of the matrix is at a certain temperature, the slower the rate of evaporation of water in the matrix with time, and the greater the water activity on the surface of the particles favoring the growth of microorganisms. However, after the matrix reaches a certain thickness, the rate of water evaporation in the matrix tends to drop; similarly, the exchange rate of air in the matrix decreases, which is not conducive to the growth of microorganisms.

The increment of CP with the increase of fermentation temperature and water–material ratio up to a critical point can be observed in Figure 4d. The elliptical nature of the contour plot in CP indicates an obvious interactive influence between fermentation temperature and water–material ratio. The effect of temperature on matrix water content is mainly caused by the evaporation of water. The higher the temperature, the faster the rate of matrix water evaporation, resulting in the decrease of matrix water content. The high specific heat capacity of water and the decrease of pores in SBM particles with the increased matrix water content exerts a combined influence on the temperature in the fermentation substrate. Temperature and water–material ratio significantly influenced the CP at the maximum degree within the experimental region. However, there was no significant interaction between these two factors on the DH in this study.

3.2.3. Validation of the Optimization Fermentation Medium

In Table 6, the observed and predicted DH and CP content are reported. Through a one-sample *t*-test, there was no significant difference between the observed value and the predicted value of DH and CP, and the predicted value falls into the 95% confidence interval. It was suggested that the errors between the predicted and verification values were to be considered small, and this means that the model is acceptable for optimization. The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point.

Table 6. Comparison between predicted and experimental responses at the optimum condition.

Response	Predicted Value	Observed Value	*p*-Value	95% Confidence Interval	
				Lower	Upper
CP (%)	55.76	55.71 ± 0.17	0.673	55.30	56.12
DH (%)	15.96	15.85 ± 0.08	0.137	15.66	16.04

CP: crude protein; DH: degree of protein hydrolysis.

3.3. Comparison of Nutritional Values of FSM and SBM

3.3.1. Nutrient Composition

Results of the nutrient analysis of SBM and FSM are shown in Table 7. The average moisture content of the air-dried FSM was significantly lower than that of the SBM feedstock by 13.7%. In this study, it was found that the FSM samples could remain powdered after crushing at a moisture content of 8.2% or less, but if the moisture percentage increased, the FSM from Bacillus subtilis would become lumpy or even sticky, which would make it impossible to mix the feed effectively.

Table 7. Nutrient and amino acid composition of SBM and FSM (% dry matter basis).

Items	SBM	FSM	*p*-Value	Change (%)
Dry matter (%)	90.56 ± 0.09	91.85 ± 0.08	<0.001	1.4
CP (%)	50.72 ± 0.30	55.71 ± 0.17	<0.001	9.8
CL (%)	2.08 ± 0.07	1.77 ± 0.05	0.004	−14.9
CF (%)	5.80 ± 0.21	4.98 ± 0.33	0.022	−14.1
CA (%)	6.33 ± 0.12	6.68 ± 0.05	0.010	5.4
PDI (% of CP)	31.20 ± 0.70	21.90 ± 0.79	<0.001	−29.8
KPS (% of CP)	80.43 ± 1.96	94.47 ± 0.81	<0.001	17.5
DH (%)	5.81 ± 0.18	16.01 ± 0.11	<0.001	175.3
Total phenol ($mg \cdot g^{-1}$)	3.25 ± 0.18	13.32 ± 1.13	<0.001	309.4
Flavonoids ($mg \cdot g^{-1}$)	1.36 ± 0.18	4.49 ± 0.27	<0.001	231.0

SBM: soybean meal; FSM: fermented soybean meal. CP: crude protein; CL: crude lipid; CF: crude fiber; CA: crude ash; PDI: protein dispersibility index; KPS: KOH protein solubility; DH: degree of protein hydrolysis.

The protein content of FSM significantly increased by 9.8% compared with SBM after 62.3 h of fermentation. Meanwhile, crude lipid and crude fiber content significantly decreased by 14.9% and 14.1%, respectively. The increase in CP is mainly due to a relative change in the loss of dry matter as a result of microbial hydrolysis and the metabolism of carbohydrates and fats as a source of energy [67]. Microbial decomposition and synthesis by *B. s. natto* barely change the total nitrogen content, but it breaks down and utilizes carbohydrates and lipids.

PDI is the percentage of total protein dispersed in water under standard conditions and is related to the amount of heat to which the SBM is exposed during production. Compared to the SBM sample, the PDI decreased from 31.20 to 21.90. It was found that the feed conversion was best for fish fed SBM with a PDI of 20, intermediate for fish fed SBM with a PDI of 11 or 70, and worst for fish fed SBM with a PDI of 88 in the study on Brown Trout, which supplemented feed with SBM, and PDI values are a useful indicator of the nutritional value of SBM in fish feed [68]. In our study, the PDI of FSM was reduced by 29.8%, suggesting that the fermentation process mitigated thermal damage during the processing of SBM. Although the optimal PDI of SBM in feeds of different fish needs further study, 20 is a PDI worthy of reference.

During the processing of SBM, heat causes FAA and other compounds to form inter and intramolecular bonds that cannot be opened by digestive enzymes, thus reducing the solubility of the protein. The KPS method has been recognized in recent years as the best method for assessing the over and under-processing of soybeans. The study by Araba and Dale indicated that the KPS values below 70% suggested overprocessed SBM, and there is a need to add lysine, arginine, or methionine, either alone or in combination, to overcome

growth inhibition in broiler chicks caused by over-processing [49]. For FSM, the detection of protein solubility is not only to evaluate the over and under-processing level of SBM but also to judge the degree of protein denaturation, detrimental for the effective utilization of protein. In our study, KPS was significantly increased by 17.4% after fermentation. This indicates that the FSM was not over-processed, and the protein was effectively degraded.

Flavonoids and phenols in fermentation products have antioxidant properties. The antioxidant properties of flavonoids are mainly achieved through the scavenging of superoxide anions [69]. The concentration of phenolic compounds in SBM also increases after fermentation, resulting in increased antioxidant and metal chelating activity [70]. The increase in phenolic acid in fermented soybeans may be due to the production of β-glucosidase during the fermentation process [71]. The results of this study showed that there was a 231.0% and 309.4% increase in flavonoids and total phenol content, respectively, in FSM. This indicates that the fermentation of *B. s. natto* increases the value of FSM in terms of antioxidant activity. Therefore, the ability of fermentation to enhance the antioxidant properties of SBM can be explored as a cost-effective way to reduce the oxidative stress of animals after the consumption of SBM.

Neutral proteases, a group of proteases with an optimum action pH between 6.0 and 7.5, are the main extracellular proteases produced by *B. subtilis* species. Neutral proteases are produced after the exponential growth period; when cultures enter the stationary phase and begin the sporulation process. The physiological role of the proteases is not well known. They are thought to play a role in spore formation, to be involved in regulating cell wall turnover, and to be scavenging enzymes [72]. Neutral proteases have high activity and play a dominant role among several proteases in hydrolyzing proteins [73]. Therefore, in the present study, the NPA over the SSF process was followed (Figure 5). The pattern of change in NPA was consistent with the different growth stages of *B. s. natto*: 0–15 h for spore germination, 15–45 h for cell growth, and after 45 h for sporulation. In the early stages of fermentation, the resting spores of *B. s. natto* germinate efficiently, and germination is favored in order to prepare for the secretion of enzymes. During the cell growth phase, *B. s. natto* is rapidly accompanied by rapid fermentation and the secretion of degradative enzymes that break down proteins, carbohydrates, etc., into absorbable micromolecules [36]. After 50 h of fermentation, the NPA decreased, probably due to water loss, reduction of substrates, and inhibition of catabolic products [74]. The results of this study confirm the importance of NPA for the growth, metabolism, and production of related enzymes of *B. s. natto*.

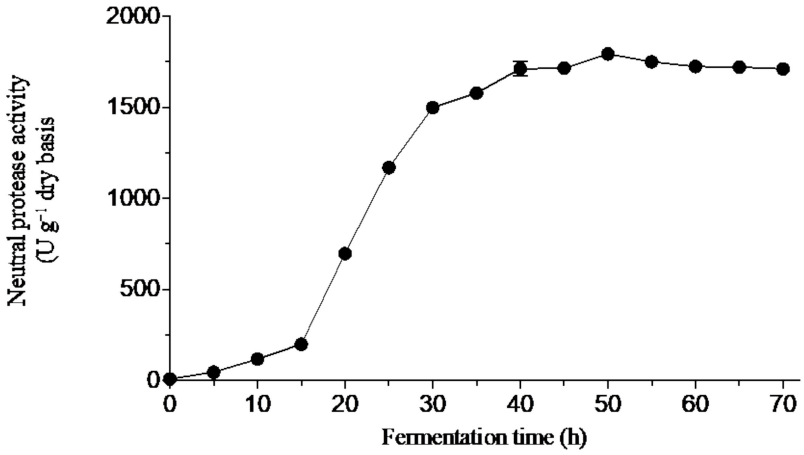

Figure 5. Neutral protease activity (NPA) as a function of SSF time.

3.3.2. Amino Acid Composition of SBM and FSM

An increase of 11.6% of the TAA content was found in FSM compared with SBM (Table 8). After the fermentation process, the EAA, including methionine, isoleucine,

valine, threonine, and tryptophan in FSM, significantly increased by 20.9%, 8.0%, 20.5%, 23.2%, and 14.9%, respectively. For non-EAA, serine, proline, and alanine contents in FSM significantly increased by 17.7%, 13.7%, and 11.2%, respectively, compared with SBM. Although fermentation concentrates the amino acids in SBM, it does not have a significant effect on the composition of amino acids.

Table 8. Amino acid composition of SBM and FSM (% dry matter basis).

Items	SBM	FSM	*p*-Value	Change (%)
EAA				
Lysine	2.93 ± 0.12	3.18 ± 0.21	0.149	8.5
Methionine	0.70 ± 0.06	0.85 ± 0.04	0.028	20.9
Isoleucine	2.37 ± 0.08	2.56 ± 0.06	0.025	8.0
Leucine	3.87 ± 0.15	4.25 ± 0.25	0.086	9.8
Valine	2.10 ± 0.11	2.53 ± 0.13	0.012	20.5
Arginine	3.54 ± 0.25	3.90 ± 0.05	0.070	10.2
Threonine	1.91 ± 0.13	2.35 ± 0.20	0.032	23.2
Tryptophan	0.65 ± 0.04	0.75 ± 0.03	0.029	14.9
Histidine	1.45 ± 0.10	1.59 ± 0.07	0.103	9.9
Phenylalanine	2.49 ± 0.15	2.74 ± 0.12	0.086	10.1
NEAA				
Cystine	0.84 ± 0.07	0.92 ± 0.02	0.157	9.1
Tyrosine	1.84 ± 0.10	2.01 ± 0.10	0.098	9.2
Serine	2.17 ± 0.11	2.55 ± 0.13	0.018	17.7
Glutamic acid	8.17 ± 0.62	9.18 ± 0.09	0.049	12.3
Proline	2.51 ± 0.14	2.85 ± 0.05	0.016	13.7
Glycine	2.00 ± 0.17	2.17 ± 0.18	0.297	8.3
Alanine	2.11 ± 0.11	2.34 ± 0.06	0.033	11.2
Aspartic acid	5.70 ± 0.20	6.05 ± 0.25	0.128	6.1
EAA	22.01 ± 0.41	24.70 ± 0.20	<0.001	12.2
NEAA	25.34 ± 0.54	28.07 ± 0.28	0.001	10.8
Total Amino Acids	47.35 ± 0.68	52.77 ± 0.11	<0.001	11.4
Total free amino acid	0.46 ± 0.00	6.33 ± 0.09	<0.001	1276.1

SBM: soybean meal; FSM: fermented soybean meal. EAA: essential amino acids; NEAA: non-essential amino acids.

The principal component analysis of amino acid of all samples (n = 30) after fermentation showed that the five amino acids with the greatest response to the fermentation variables (PC1) were valine, proline, serine, alanine, and threonine, in descending order, and where valine and threonine belong to EAA (Figure 6). However, fermentation did not significantly increase the content of lysine, regarded as one of the most limiting amino acids in fish feed.

After fermentation, all hydrolyzed amino acids increased. The increase of 12.2% of total EAA was higher than the increase of 10.8% of the total NEAA in FSM. Sarkar et al. reported a similar result [75]. The increased EAA of FSM produced with the *B. s. natto* is considered to be a good source of protein for aquafeed. Sulfur-containing amino acid methionine was improved by fermentation with *B. s. natto* in this study, which was the most critical amino acid in soya but was not improved with *B. subtilis* in some previous studies [76,77]. *B. s. natto* will be a microorganism for SBM fermentation that can produce methionine for the aqua-feed industry in the future.

The profile and content of FAA in FSM obviously increased with increasing total FAA content at 12.76-fold compared with the SBM. This may be due to the proteolytic activity of *B. s. natto*. Sarkar et al. showed that fermentation of soybeans with *B. subtilis* led to a 60-fold increase in FAA [75].

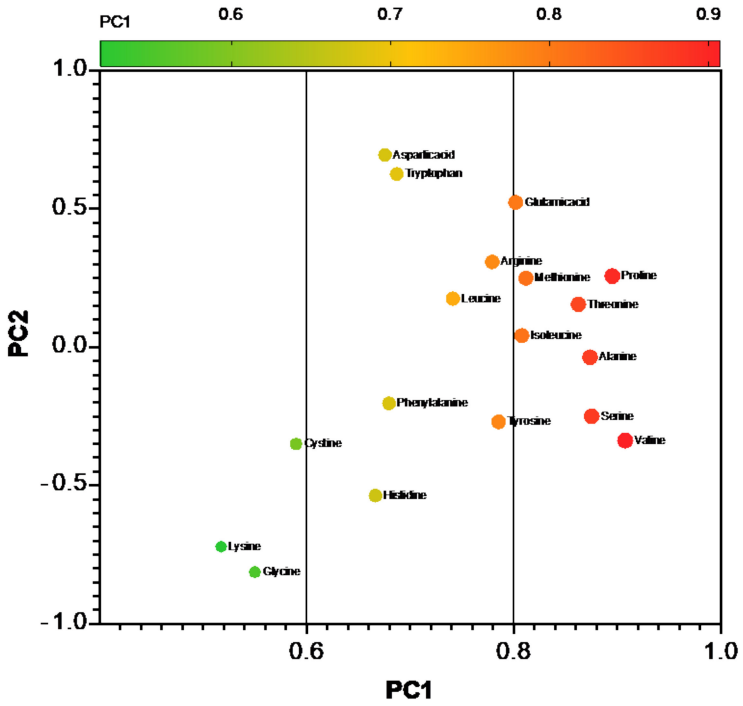

Figure 6. The principal component analysis loadings of amino acid changes between FSM and SBM. The PC1 and PC2 axis explained 59.31% and 17.58% of the total variation, respectively. PC1 is mainly generated by fermentation treatment; PC2 is caused by systematic and analytical errors. Higher amino acid PC1 scores indicate greater influence by fermentation.

3.3.3. ANFs Composition

SBM contains a variety of ANFs. Protein-based ANFs include trypsin inhibitors, lectins, soybean allergens, and fat oxidase, while carbohydrate-based ANFs include soybean oligosaccharides (mainly includes raffinose and stachyose) and non-starch polysaccharide. Phytic acid and saponin are secondary metabolite anti-nutritional factors. Many studies have shown that antigens in soy and soy products are the main cause of allergic reactions in fish, causing gastrointestinal damage and digestive disorders [77].

Table 9 shows that the content of ANFs in FSM is lower than that in SBM samples before fermentation. The average content of trypsin inhibitor decreased by 53.7%, lectins decreased by 99.4%, glycinin decreased by 77.7%, β-conglycinin decreased by 71.6%, while the average content of stachyose decreased by 95.4%, raffinose decreased by 90.9%, and the average content of phytic acid decreased by 92.6%.

Table 9. Anti-nutritional factors (ANFs) of SBM and FSM ($mg \cdot g^{-1}$).

Items	SBM	FSM	*p*-Value	Change (%)
Trypsin inhibitor	3.50 ± 0.10	1.62 ± 0.09	<0.001	−53.67
Glycinin	79.20 ± 0.44	17.63 ± 1.68	<0.001	−77.74
β-Conglycinin	106.38 ± 4.15	30.17 ± 6.91	<0.001	−71.64
Lectins	3.35 ± 0.20	0.02 ± 0.01	<0.001	−99.40
Raffinose	18.43 ± 1.05	1.67 ± 0.23	<0.001	−90.94
Stachyose	11.92 ± 1.55	0.55 ± 0.08	<0.001	−95.38
Phytic acid	1.73 ± 0.11	0.13 ± 0.01	<0.001	−92.62

SBM: soybean meal; FSM: fermented soybean meal.

Protease inhibitor is the most important ANF in soybean. Trypsin inhibitory factor is a crystalline globulin that forms a stable complex with trypsin and deactivates the protease, thus decreasing the digestibility of protein in feed and reducing the availability of amino acids, resulting in growth inhibition. At the same time, the trypsin inhibitory factor can

cause increased pancreatic secretion activity, resulting in the overproduction of trypsin and chymotrypsin. Because these proteins are rich in sulfur-containing amino acids, they can convert the amino acids originally used to synthesize tissue proteins to synthesize proteases, form complexes with inhibitors, and finally be excreted through feces, resulting in a large loss of endogenous nitrogen and sulfur-containing amino acids. Due to the lack of sulfur-containing amino acids in soybeans, the amino acids in the feed are not balanced, thus hindering the growth of fish. In the study of common carp by Anne [78], it was found that with the increase of the amount of fish meal replaced by soybean protein, the protease activity in the intestine and hepatopancreas decreased significantly.

Lectins are defined as proteins that bind to carbohydrates. The same features that lectins use to defend plants in nature may cause problems during animal digestion. They resist being broken down in the gut and are stable in acidic environments, features that protect lectin-containing plants in nature [79]. Soybean lectin adhesive binds to the small intestinal wall, which will affect the nutritional absorption of intestinal epithelial cells, resulting in changes in the activity of digestive enzymes, the loss of endogenous protein secretion, the increase of mucin, and other related activities. Studies have shown that the integrity of the hindgut villi of Atlantic salmon fed diets containing 3.5% soy lectin was significantly disrupted, as evidenced by mucosal detachment into the intestinal lumen, cellular infiltration into the lamina propria, and soy lectin binding to the epithelial cells of the small intestinal villi [80].

The antigenic proteins in SBM consist of two main types: glycinin (11S) and β-conglycinin (7S), which account for 70% of the total antigenic protein, with the 11S component being essentially a single protein, accounting for 25–35% of the total soybean protein, and the 7S component accounting for 30–35% [81]. The alpha subunit of β-conglycinin is one of the main allergens found in soybeans. The immune damage caused by allergic reactions to soybean antigenic proteins is mainly in the intestinal tract. Studies on the mechanisms of intestinal sensitization of fish by soy antigenic proteins are scarce. Soybean allergens can also damage the structure of the digestive organs when soy protein replacement in fish feed is increased to a certain level.

Soybean oligosaccharide or α-galactosides is the general name of soluble oligosaccharides in SBM; the total content in SBM is 12–15%, of which 5–6% is stachyose, 1–2% is raffinose, and 6–7% is sucrose [82]. The negative effect on fish may be due to the combination with bile acids or blocking the action of digestive enzymes and the movement of substrates in the intestine [83]. Soy carbohydrates led to reduced nutrient utilization in Atlantic salmon; however, soy oligosaccharides had little effect on protein utilization [84].

The presence of phytic acid in the diet can negatively affect the growth of commonly farmed fish, such as carp, tilapia, trout, and salmon. Due to the structural characteristics of phytic acid phosphorus itself and its strong chelation, phosphorus in the form of phytate cannot be used by fish; phytic acid also affects the utilization of other nutrients. Phytic acid can chelate with bivalent (Zn^{2+}, Ca^{2+}, Mg^{2+}) or trivalent (Fe^{3+}) metal ions to form insoluble complexes, thus reducing the biological potency of these metal ions. Fermentation has been shown to reduce the phytic acid content of grains due to the action of phytase produced by yeast or lactic acid bacteria [85]. In our study, *B. s. natto* was also found to be effective in reducing the phytic acid content.

Based on the results of the reduction in the content of various ANFs in FSM, the feasibility of further increasing the content of SBM in aquatic feed is provided.

3.3.4. SDS-PAGE Analysis

Figure 7 shows the protein profiles of SBM and FSM analyzed by SDS-PAGE. SBM electrophoresis lanes show almost all soy protein bands, including α'-subunit (78 kDa), α-subunit (70 kDa), and β-subunit (47 kDa) of β-accompanying soy globulin, as well as the acidic subunits (32 kDa) and basic subunits (19 kDa) of soy glycinin. However, the FSM electrophoresis lanes only show a large number of proteins <25 kDa. All the β-conglycinin and 32 kDa glycinin in FSM samples were completely digested. However, there are still a

few shadow bands of the 19 kDa glycinin, indicating that there is still a small amount of 19 kDa glycinin remaining in FSM.

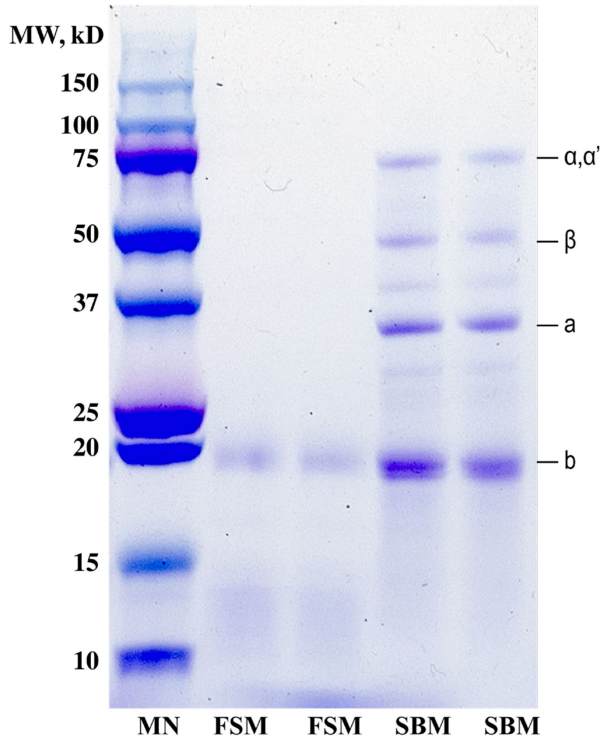

Figure 7. SDS-PAGE profile of SBM before and after SSF. MN: standard molecular-weight marker; FSM: fermented soybean meal; SBM: unfermented soybean meal; α': α'-subunit; α: α-subunit; β: β-subunit; a: acidic subunits; b: basic subunit.

It can be seen from the result that soybean protein has changed tremendously after fermentation, especially the content of anti-nutritional macromolecular proteins (glycinin, β-conglycinin, etc.), which decreases significantly after fermentation. The microbial fermentation of SBM not only degraded the anti-nutritional globulin but also broke down almost all the large molecules of SBM proteins, and the FSM mainly contained small molecule proteins or small molecule peptides with a molecular weight below 22 kDa.

SDS-PAGE analysis showed that FSM proteins, including the major soybean allergens, were hydrolyzed by *B. s. natto* after fermentation. Similar results were found for FSM from *B. subtilis* or other microorganisms [86,87]. *B. s. natto* produces a large number of extracellular proteases that secrete and degrade proteins in the culture medium [88].

4. Conclusions

This study demonstrates the feasibility of RSM to optimize the SSF technology of SBM for the widespread use of FSM in aquatic and livestock feeds and the effective enhancement of the nutritional value of SBM that can be achieved by *B. s. natto* under optimized fermentation parameters. The results showed that fermentation temperature 43.82 °C, fermentation time 62.32 h, water–material ratio 1.08, and SBM layer thickness 2.02 cm were the most suitable fermentation conditions for obtaining high protein content and DH. The FSM had low anti-nutritional factor levels, higher protein levels, higher free amino acid levels, relatively high antioxidant activity, and NPA. Given the desirable protein quality of the FSM, the significant reduction in anti-nutrients, and the cost-effectiveness of the process, SSF in general and SSF with *B. s. natto* in particular show great potential for the improvement of plant-based ingredients for aquafeed.

Based on this study, we will further conduct feeding experiments with different aquatic animals to provide data to support the amount of *B. s. natto* FSM to be used in aquatic feed.

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