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Production of Nattokinase from Hemp Seed Meal by Solid-State Fermentation and Improvement of Its Nutritional Quality

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Abstract: Fermented foods have gained immense popularity in recent years due to their distinct flavor profile. Given the increasing demand, there is a growing focus on optimizing their nutritional quality while also reducing their costs. In this study, using a novel approach, hemp seed meal was utilized as a solid fermentation substrate to produce nattokinase (NK). Using a combination of one-factor-at-a-time experiments, Plackett–Burman design, and Box–Behnken design, the optimal fermentation conditions of *Bacillus subtilis* 13932 (NK-producing strain) were determined. The initial ratio of HSM (hemp seed meal) to water was 1:2.0 (*v*:*w*), the thickness of the substrate was 2.9 cm, the bacterial inoculum volume was 10% (*v*:*w*), the relative humidity was 75.2%, the temperature was set at 35 °C, and the fermentation time was 20 h. The NK activity under these conditions was measured to be 7067.12 IU/g. During fermentation, 15.15% of soluble peptides were produced, which exhibited hydroxyl radical removal ability and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical removal ability of 14.85%, down from 32.96%. Furthermore, trypsin inhibitor and urease in HSM decreased by 42.6% and 73.6%, respectively, improving the nutritional quality of HSM. Sensory evaluations indicated that HSM is expected to be a popular food, highlighting the potential of using HSM as a solid fermentation substrate for NK production.

Keywords: nattokinase; hemp seed meal; solid-state fermentation; antioxidant; anti-nutritional factors

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1. Introduction

Hemp seed meal (HSM) is a high-value byproduct obtained after oil extraction from hemp seeds. Hemp seeds are rich in protein, carbohydrates, oil, insoluble fiber, and minerals [1]. The are also a good source of phytonutrients, such as tocopherols, carotenoids, and sterols [2]. HSM contains a full complement of recommended amino acids required for the development of infants and children, and the protein content of HSM is not only abundant, but also highly digestible [3,4]. Traditionally, HSM was added directly to animal feed, causing a waste of valuable nutritional resources [5]. In previous studies, researchers have attempted to add HSM during the preparation of bread and biscuits to produce foods suitable for a wider range of people [6,7]. However, recent studies have shown that HSM can be used as a substrate or supplement in solid-state fermentation (SSF), providing the nutritional support of carbon and nitrogen sources [8]. Nissen et al. found that HSM could be used as a substrate for beneficial lactic acid bacteria, producing fermented HSM with more bioactive substances, such as organic acids and terpenes, which could be applied as a prebiotic [9].

Fermented foods are popular in Asian countries due primarily to their unique flavors. Nattokinase (NK) is a fibrinolytic protease found in the traditional Japanese fermented food natto, made from soybeans fermented with *Bacillus* [10]. Research has demonstrated that the strong fibrinolytic activity of NK is retained after intestinal absorption with high enzymatic

specificity and minimal side effects [11]. NK is a potential thrombolytic drug with high efficiency, safety, and economy, leading to widespread interest in its application. Soybeans, chickpeas, and wheat bran are commonly used as fermentation substrates for NK [12–14]. In addition to traditional fermentation sources, current NK research has been focused on the ongoing quest for low-cost fermentation substrates with rich flavor and higher nutritional quality. Wang et al. used shrimp shells as a substrate and inoculated *Pseudomonas* sp. to produce NK, effectively reducing the production cost of NK [15]. Li et al. replaced the nitrogen source in the fermentation medium with tofu processing wastewater and observed a 47.89% increase in NK activity after optimizing the culture conditions [16]. Guo et al. used Ginkgo seeds as a substrate and achieved a fibrinolytic activity of 3682 ± 43 U/g, and the fermented Ginkgo seeds had higher total flavonoid and lower ginkgolic acid contents [17]. Dong et al. fermented chestnuts with *Bacillus natto* and obtained highly active NK. Furthermore, the antioxidant activity and α-glucosidase inhibition activity were also increased [18].

Solid-state fermentation is a simpler and less costly and energy-intensive method of producing NK, with the advantages of high production efficiency, good product stability, and environmental friendliness [19]. Another advantage is that beneficial byproducts, such as valuable fermentation substrates, are more readily produced by solid-state fermentation.

B. subtilis, the strain most commonly used for NK production, is biosafe and has a high protein secretion capacity [20,21]. The rich protease system of B. subtilis is able to break down large protein molecules in the substrate into small polypeptides, facilitating the uptake and use of the nitrogen source by the bacterium. B. subtilis strain 13,932 was previously shown to produce highly active NK in the medium when tofu processing wastewater was used as the nitrogen source [16]. Numerous studies have demonstrated that peptides derived from hemp protein exhibit many beneficial properties, including regulating hypertension, antioxidation, preventing platelet aggregation, modulating the immune system, reducing cholesterol, and possessing antibacterial properties [22]. Furthermore, the fermentation of HSM using B.subtilis is anticipated to produce active polypeptides alongside NK.

The aim of this study was to investigate the feasibility of using HSM as a novel solid-state fermentation substrate and to explore the optimum conditions for NK production via solid-state fermentation of HSM by *B. subtilis* 13,932. At the same time, we wanted to obtain HSM peptides with antioxidant activity and reduce the anti-nutritional factors of HSM via solid-state fermentation. This innovative method aims to create a novel, highly active NK substrate, reducing the overall manufacturing cost while simultaneously providing an efficient and eco-friendly means of utilizing HSM for high-value applications.

2. Materials and Methods

2.1. Materials and Reagents

The HSM used in this study was obtained by crushing leftover hemp seed waste after shelling and oil extraction (particle size range: 0.6–4.0 mm), which was purchased from Hongtian Jiali Co., Ltd. (Jinzhong, China). Thrombin (1000 U) and fibrinogen were purchased from Beijing Zhongke Quality Control Biotechnology Co., Ltd. (Beijing, China). Agarose was purchased from Sigma-Aldrich (St Louis, MO, USA), and other analytical reagents were purchased from Nanjing WANQING Chemical Glassware and Instrument Co., Ltd. (Nanjing, China).

2.2. Microorganism and Culture

B. subtilis 13,932 (Conservation No. CGMCC13932) was maintained in our laboratory. The strain was activated using LB medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.4). The activated strain was then transferred to a seed medium and incubated at 37 °C for 16 h prior to being transferred onto a solid medium. The seed medium included 20 g/L glucose, 20 g/L soybean protein, 0.2 g/L $CaCl_2$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L K_2HPO_4, and 1 g/L KH_2PO_4, with the pH adjusted to 7.4.

2.3. Determination of HSM Composition

The primary components of HSM were determined using the Chinese national standard analytical techniques, protein (GB 5009.5-2016), fat (GB 5009.6-2016), dietary fiber (GB 5009.88-2014), moisture (GB 5009.3-2016), and amylum (starch) (GB 5009.9-2016).

2.4. Solid-State Fermentation of Nattokinase by HSM

2.4.1. One-Factor-at-a-Time Experiments (OFAT)

A 100 g sample of HSM was soaked in distilled water for 8 h and sterilized at 121 °C for 20 min, according to the initial ratio of HSM to water (1:1, 1:1.5, 1:2, 1:2.5, 1:3) (*w*:*v*). After cooling, the fermentation substrate at a prescribed thickness (1 cm, 2 cm, 3 cm, 4 cm, 5 cm) was prepared, and the *B. subtilis* 13,932 seed medium was added according to the tested bacterial inoculum volumes (4%, 6%, 8%, 10%, 12%) (*w*:*v*). The mixture was evenly mixed and spread onto the sterilization tray. The relative humidity of the fermentation tank was set at a precise level (60%, 65%, 70%, 75%, 80%), and the temperature was controlled constantly (25 °C, 30 °C, 35 °C, 40 °C, 45 °C) throughout the fermentation. The mixture was mixed every 4 h, and the fermentation was carried out for a defined length of time (12 h, 16 h, 20 h, 24 h, 28 h).

2.4.2. Plackett–Burman Design (PB Design)

Based on the results of our OFAT experiments, the PB design sought to determine the optimal conditions for the fermentation process. Specifically, it selected six factors and investigated the initial ratio of HSM to water (A), thickness of the substrate (B), bacterial inoculum volume (C), relative humidity (D), temperature (E), and fermentation time (F) (coding levels shown in Table 1). The response value chosen for investigation was the NK activity, and the experiment aimed to examine the impact of each factor on the fermentation results.

Table 1. Minimum and maximum range of each parameter selected in PB design.

Factor	Symbol	Level	
		Low	High
Initial ratio of HSM to water (*v*:*w*)	A	1:1.5	1:2.5
Thickness of the substrate (cm)	B	2	4
Bacterial inoculum volume (*v*:*w*)	C	8	12
Relative humidity (%)	D	70	80
Temperature (°C)	E	30	40
Time (h)	F	16	24

2.4.3. Box–Behnken Design (BBD)

Based upon the results of our PB design, the four factors A, B, D, and E were identified as the key factors impacting fermentation. Acknowledging these factors, BBD was carried out with NK activity as the response (coding levels shown in Table 2).

Table 2. Coded and real values of variables in the BBD.

Factor	Symbol	Level		
		Low	0	High
Initial ratio of HSM to water (*v*:*w*)	A	1:1.5	1:2	1:2.5
Thickness of the substrate (cm)	B	2	3	4
Relative humidity (%)	D	70	75	80
Temperature (°C)	E	30	35	40

2.5. Extraction of NK and Determination of Enzyme Activity

To prepare the sample, 10 g of fermented hemp seed meal (FHSM) and 40 mL of 0.9% NaCl solution were mixed in a 250 mL flask and mixed at 100 rpm for 1 h. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected for analysis of NK

enzyme activity using the fibrinolytic plate method [16]. To prepare the agarose solution, solid agarose was dissolved in 100 mL of a mixed solution containing phosphate buffer solution (pH = 7.8) and 0.9% NaCl solution at a ratio of 1:17. The solution was then sterilized at high temperature and kept at 60 °C. To prepare the solid plates, 68.67 mL of 1.5 mg/mL fibrinogen solution and 5.28 mL of 1 bp/mL thrombin solution (dissolved in 0.9% NaCl solution) were preheated and added to the agarose solution. The mixture was then thoroughly mixed and prepared in solid plates, which were then punched after coagulation. For analysis, 15 µL of the supernatant was added into the well of the solid plate, which was then cultured at 37 °C for 16–18 h. The diameter of the fibrinolytic ring was then measured. The enzyme activity of the urokinase standard solution was taken as the ordinate, and the product of the diameter of the fibrinolytic ring produced by urokinase under the same operation was taken as the abscissa, in order to create a standard curve.

2.6. Determination of Soluble Peptide Content

The method for the determination of soluble peptide content is based upon the method of Benjakul et al. [23]. A 1 g sample of FHSM was thoroughly homogenized with 10 mL of water. The homogenization solution was mixed with an equal volume of 10% (*w*/*v*) trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 10 min after allowing it to stand for 30 min to remove macromolecular proteins. The nitrogen content was measured using a total nitrogen analyzer. The TCA soluble peptide content percentage was calculated as follows:

TCA soluble peptide content (%) = nitrogen content in supernatant after TCA precipitation/nitrogen content in homogenization solution × 100

2.7. Detection of Antioxidant Activity

The hydroxyl and DPPH radical scavenging abilities were analyzed using kits produced by Jiangsu Addison Biotechnology Co., Ltd. (Yancheng, China).

2.8. Determination of Anti-Nutritional Factors

Urease levels were measured using a Keming Biotechnology Co., Ltd. kit. Urease enzyme activity was defined as 1 µg NH3-N per minute produced per g of tissue is one enzyme activity unit. Trypsin inhibitor activity was determined using an ELISA kit purchased from Jiangsu Meimian Industrial Co., Ltd. (Yancheng, China).

2.9. Sensory Characteristics

The sensory properties of FHSM have been modified from a protocol established by Feng et al. [24]. The sensory evaluation team was composed of 10 people with sensory evaluation experience. The prepared FHSM was scored from 1 to 5 points for its appearance, smell, texture, taste, and stringiness (5 = very good, 4 = good, 3 = acceptable, 2 = dislike, 1 = highly dislike).

2.10. Statistical Analysis

All tests were performed in triplicate. The data are presented as mean ± standard deviation (SD). Design-Expert 8.0.6 software was used in the PB Design and BBD, as well as for corresponding data analysis, while Origin 2018 software was used for graph construction. The significance threshold values of the obtained data were set at $p < 0.05$.

3. Results and Discussion

3.1. Components of HSM

The traditional fermentation substrate of NK is soybeans, which have a protein content of about 40% protein and a fat content of around 20%, with a small amount of amylum (4.66%–7%) and higher amounts of hemicellulose and pectin [25]. However, compared to soybeans, the oil extraction waste HSM has a significantly smaller amount of fat residue (1.8 ± 0.02 g/100 g) (Table 3). Instead, HSM is dominated by amylum

(56.4 ± 1.17 g/100 g) and protein (31.9 ± 0.91 g/100 g), in addition to a small amount of moisture (7.91 ± 0.08 g/100 g) and dietary fiber (1.16 ± 0.05 g/100 g). Because of its high carbon and nitrogen content, HSM is an ideal source for *B. subtilis* growth and NK synthesis in SSF.

Table 3. Main components of HSM.

Component	Moisture	Protein	Fat	Amylum	Dietary Fiber
Content (g/100 g)	7.91 ± 0.08	31.9 ± 0.91	1.8 ± 0.02	56.4 ± 1.17	1.16 ± 0.05

3.2. One-Factor-At-A-Time Experiments

SSF has gained significant attention from the scientific community in recent years for industrial biological development due to its ability to reduce energy demand and minimize wastewater generation, aligning with the trends of environmental protection [26]. Various factors impact solid-state fermentation, including pH, temperature, moisture, water activity, substrate, inoculum, aeration, agitation, nutrients, and the effect of sterilization [27]. This study examined the effects of six factors, including the initial ratio of HSM to water; the substrate thickness, bacterial inoculum volume, relative humidity, temperature, and time were chosen to evaluate their effects on the biomass and NK activity.

The degree of aqueous substrate plays a crucial role for the growth and enzyme production of bacteria. Low water content restricts bacterial growth, resulting in reduced NK activity, while high water content impedes gas flow and increases the risk of infection [27]. At an initial ratio of HSM to water of 1:2 (*w*:*v*), the highest species biomass was 229.3 ± 9.6 × 10^7 CFU/g, and NK activity also reached a maximum of 6106.7 ± 178.8 IU/g (Figure 1a). Figure 1b illustrates the effect of the solid fermentation substrate thickness on NK activity and that both the bacterial biomass and NK activity were highest when the substrate thickness was 3 cm. Aeration during fermentation was ineffective when the fermentation substrate was too thick; the aeration effect during fermentation was not good, affecting the bacterial biomass and NK activity. This is consistent with the findings of Bui et al., who used soybean as a substrate to produce NK, where the enzyme activity of crude NK was highest (16.55 ± 0.06 specific activity/mL) when the substrate thickness was 3 cm [28]. Figure 1c illustrates the effect of the bacterial inoculum volume on NK activity, ranging from 4% to 10%. Both the NK activity and bacterial biomass improved as the inoculum volume increased, but the bacterial biomass did not change significantly as the inoculum volume further increased, resulting in lower enzyme activity due to nutrient depletion [29]. An appropriate inoculum volume can reduce the culture cost and shorten the fermentation cycle of microorganisms. The optimal inoculum volume in this work was 10% (*v*:*w*), and NK activity reached 6785.7 ± 141.9 IU/g. Low relative activity causes water in the substrate to distribute unevenly, which affects bacterial growth. In a study by Guo et al., where Ginkgo seeds were used as the fermentation substrate, the highest fibrinolytic activity was observed at a relative humidity of 80%, 3422 ± 39 IU/g (Guo et al., 2019). Figure 1e shows the effect of temperature on NK activity, with NK demonstrating the highest activity at 37–41 °C, similar to the fermentation of *B. subtilis* 13,932 in liquid [16]. Figure 1f illustrates the effect of fermentation time on NK activity. NK activity increased with the bacterial biomass at 12–18 h, but at 24 h, the bacterial biomass further increased, while NK activity decreased. Prolonged fermentation time can reduce enzyme activity, as observed in other solid-state fermentation enzyme production results. Thus, the optimal fermentation time for producing NK was 20 h.

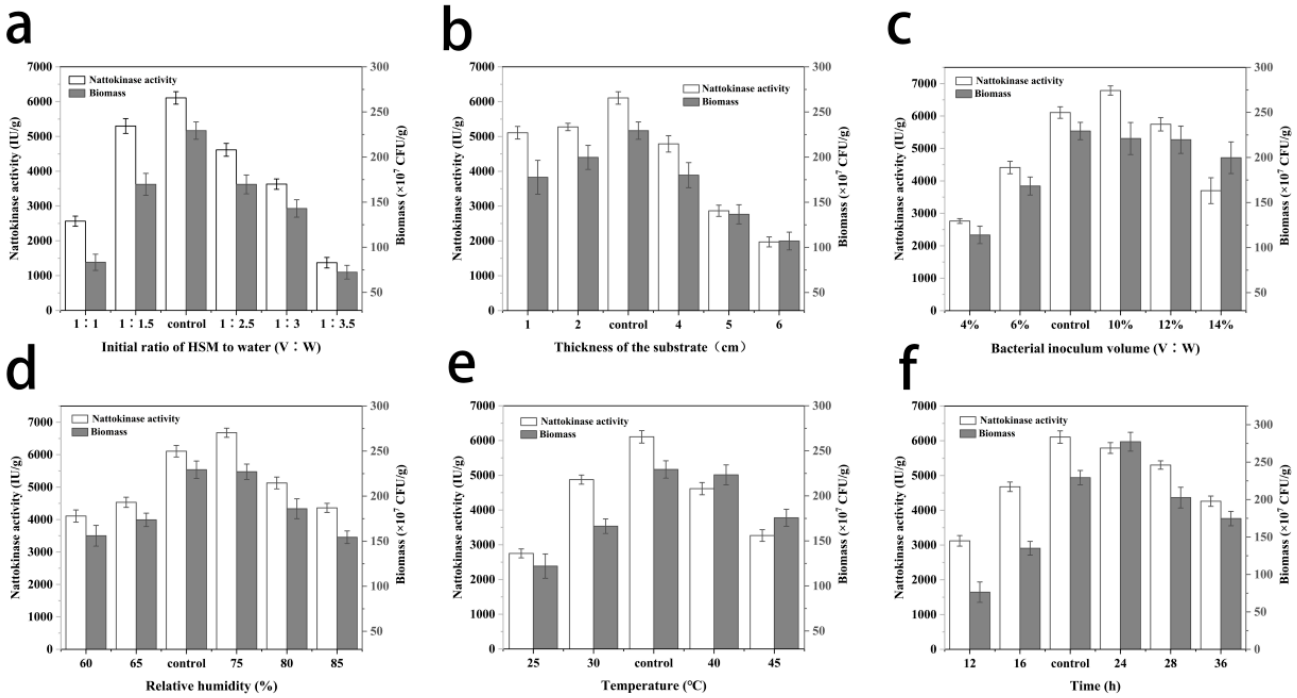

Figure 1. Effects of (**a**) Initial ratio of HSM to water (*v*:*w*), (**b**) Thickness of the substrate (cm), (**c**) Bacterial inoculum volume (*v*:*w*), (**d**) Relative humidity (%), (**e**) Temperature (°C), (**f**) Time (h) on NK activity of FHSM. Each value represents the mean ± SD (n = 3).

3.3. PB Design

The initial ratio of HSM to water (A), the thickness of the substrate (B), the bacterial inoculum volume (C), the relative humidity (D), the temperature (E), and the fermentation time (F) were selected for suitable PB design (Table 4, Supplementary Materials Table S1) based on the findings of the OFAT experiment.

Table 4. Experimental analysis of variance-response values based on nattokinase activity.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	7.317×10^5	6	1.219×10^5	10.06	0.0114	significant
A	1.296×10^5	1	1.296×10^5	10.69	0.0222	*
B	3.869×10^5	1	3.869×10^5	31.92	0.0024	**
C	23,994.96	1	23,994.96	1.98	0.2184	
D	84,101.76	1	84,101.76	6.94	0.0463	*
E	1.054×10^5	1	1.054×10^5	8.70	0.0319	*
F	1756.92	1	1756.92	0.1450	0.7190	
Residual	60,591.01	5	12,118.20			
Cor Total	7.923×10^5	11				

* Significance code: $p < 0.05$ and ** Significance code: $p < 0.01$.

The *p*-values yielded by the ANOVA were less than 0.05, indicating that the variance is significant and is fit appropriately by this test. We found that the initial ratio of HSM to water (A), thickness of the substrate (B), relative humidity (D), and temperature (E) all had significant effects on the NK activity ($p < 0.05$). The thickness of the fermentation substrate (B) had a highly significant effect on NK activity ($p < 0.01$). The following is the sequence of factors that affect NK, from greatest to least: B > A > E > D.

3.4. BBD

BBD (Table 5, Supplementary Materials Table S2) was performed on the four factors identified in the PB design to impact NK activity, namely, the initial ratio of HSM to water (A), the thickness of the substrate (B), the relative humidity (D), and the temperature (E).

Table 5. Analysis of variance for response surface quadratic model results.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	1.668×10^6	14	1.191×10^5	19.61	<0.0001	significant
A	13,240.16	1	13,240.16	2.18	0.1619	
B	62,107.24	1	62,107.24	10.23	0.0064	*
D	1297.92	1	1297.92	0.2137	0.6510	
E	821.71	1	821.71	0.1353	0.7185	
AB	4489.00	1	4489.00	0.7392	0.4044	
AD	13,665.61	1	13,665.61	2.25	0.1558	
AE	41,575.21	1	41,575.21	6.85	0.0203	*
BD	16,218.02	1	16,218.02	2.67	0.1245	
BE	29,790.76	1	29,790.76	4.91	0.0439	*
DE	229.52	1	229.52	0.0378	0.8487	
A^2	1.130×10^6	1	1.130×10^6	186.01	<0.0001	**
B^2	5.112×10^5	1	5.112×10^5	84.17	<0.0001	**
D^2	2.770×10^5	1	2.770×10^5	45.61	<0.0001	**
E^2	35,695.42	1	35,695.42	5.88	0.0295	*
Residual	85,024.08	14	6073.15			
Lack of Fit	68,251.07	10	6825.11	1.63	0.3379	not significant
Pure Error	16,773.01	4	4193.25			
Cor Total	1.753×10^6	28				
R^2	0.9515					

* Significance code: $p < 0.05$ and ** Significance code: $p < 0.01$.

As shown in Table 5, all data were fitted to a second-order polynomial model. The significance of the model was obvious by the low p value ($p < 0.0001$), and the lack of fit of the model was not significant, suggesting that the model was credible. The resulting R^2 value was 0.9515, indicating that the experimental data were well aligned with the predicted NK activity for the model. The second-order polynomial equation related to NK activity was:

$$Y = -53{,}701.62333 + 6485.48667 \times A + 2097.47833 \times B + 1244 \times D + 261.871 \times E - 67 \times A \times B + 23.38 \times A \times D - 40.78 \times A \times E - 12.735 \times B \times D + 17.26 \times B \times E - 0.303 \times D \times E - 1669.28 \times A^2 - 280.7325 \times B^2 - 8.2658 \times D^2 - 2.9673 \times E^2$$

According to the model equation Y, the linear terms A, B, D, and E and the cross-product term AD had a positive impact on the NK activity, while the cross-product terms AB, AE, and BD and the quadratic terms A^2, B^2, D^2, and E^2 had a negative effect.

As shown in Figure 2, alongside the increase of the initial ratio of HSM to water, thickness of the substrate, relative humidity, and temperature, the activity of NK had an initial decrease followed by an increase. The 3D response surfaces all showed a convex trend, indicating that NK activity (y) had its maximum value in the range of values indicated by variable (x). Analysis of the model using Design Expert software resulted in the following optimal fermentation conditions for NK production in the solid-state fermentation of *B. subtilis* 13,932: an initial ratio of HSM to water 1:1.984, a substrate thickness 2.869 cm, relative humidity 75.205%, temperature 34.995 °C. Under these conditions, the predicted NK activity was 7100.886 IU/g. After adjustment, we selected an initial ratio of HSM to water: 1:2.0, thickness of the substrate: 2.9, bacterial inoculum volume: 10%, relative

humidity: 75.2%, temperature: 35 °C, and incubation time: 20 h to obtain NK activity of 7067.12 IU/g, which was near to the predicted value.

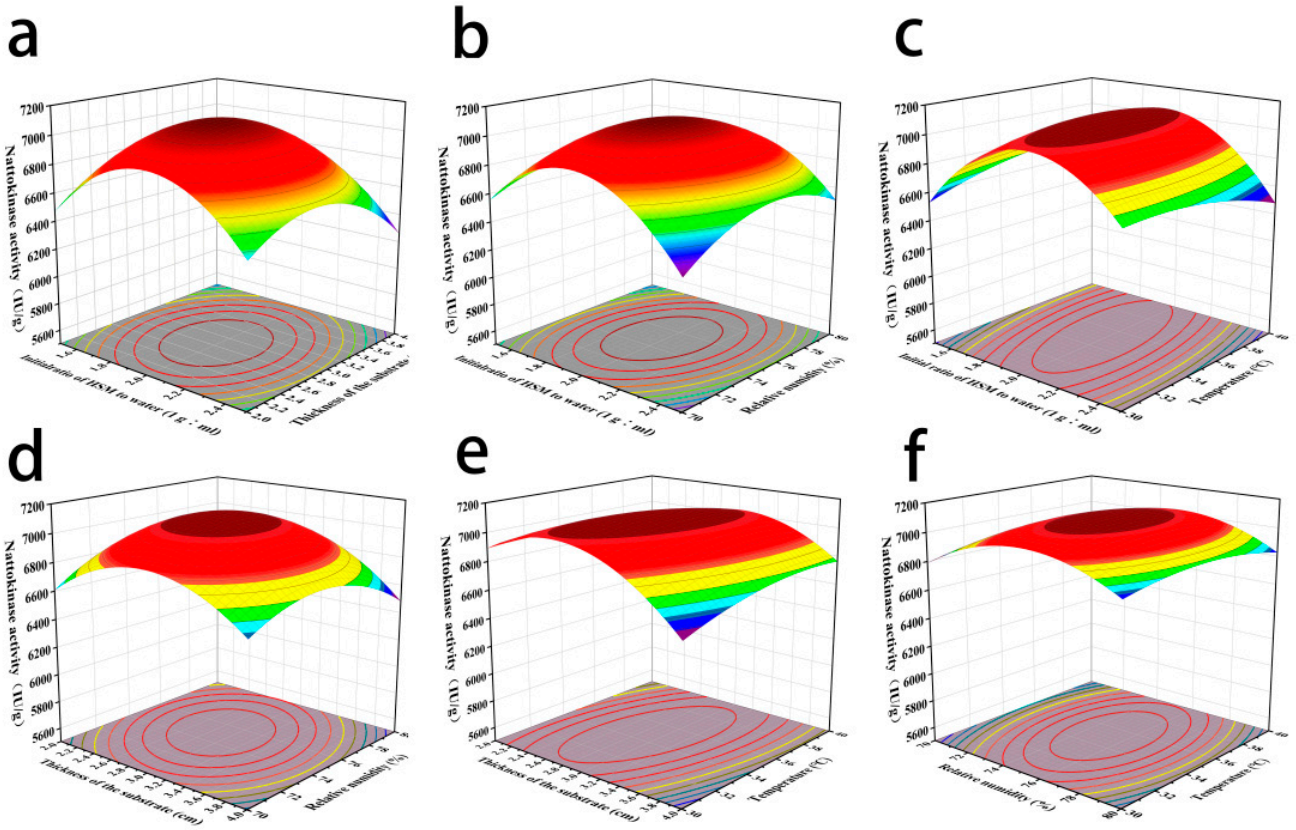

Figure 2. Response surface plot for NK production of FHSM: (**a**) initial ratio of HSM to water and thickness of the substrate, (**b**) initial ratio of HSM to water and relative humidity, (**c**) initial ratio of HSM to water and temperature, (**d**) thickness of the substrate and relative humidity, (**e**) thickness of the substrate and temperature, (**f**) relative humidity and temperature.

Table 6 illustrates the substrates, strains, and enzyme activities of NK solid-state fermentation over recent studies. *B. subtilis* and *Pseudomonas* are the predominant species for NK production. The substrates of NK solid-state fermentation were diverse, ranging from traditional soybeans, chickpeas, and wheat bran to shrimp shell, ginkgo seeds, and chestnuts. Suitable substrates effectively improved NK activity with knock-on beneficial effects, and we believe that the application of HSM will provide a promising new process for NK production.

Table 6. Substrates, strains, and NK activities in solid-state fermentation.

Substrates	Strains	NK Activity	Reference
soybeans	*Bacillus subtilis natto*	1388 U/g	[30]
soybean, rice husk	*Bacillus subtilis*	2503.4 IU/gs	[13]
chickpeas	*Bacillus subtilis* LSSE-22	39.28 FU/g	[14]
soybean residue	*Bacillus subtilis* GXA-28	986 U/g	[31]
shrimp shell	*Pseudomonas* sp TKU015	2.3 FU/mL	[15]
ginkgo seeds	*Bacillus natto* (no. 1A752)	3682 ± 43 IU/g	[17]
chestnut	*Bacillus natto*	6479 IU/g	[18]
hemp seed meal	*Bacillus subtilis* 13,932	7067.12 IU/g	This study

3.5. Contents of Soluble Polypeptides and Antioxidant Activities

HSM was fermented according to the optimal fermentation conditions described above. HSM not inoculated with *B. subtilis* under the same conditions was subjected to the content determination of soluble polypeptides and antioxidant assays. The results are shown in Figure 3.

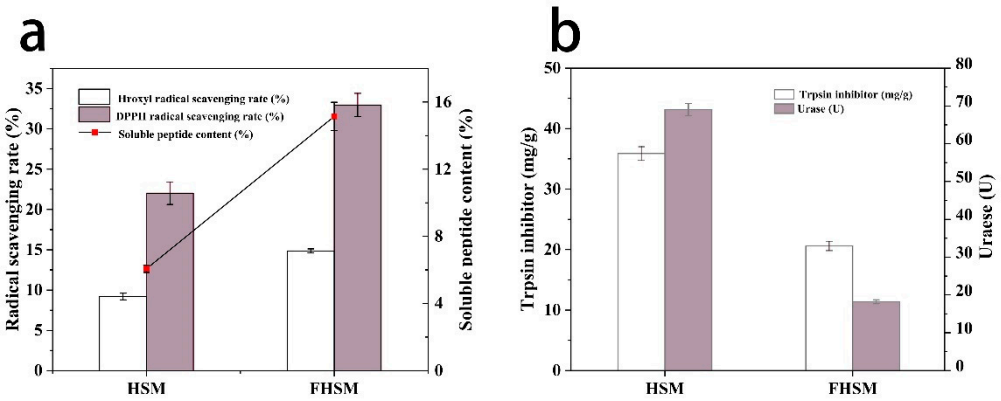

Figure 3. (**a**) Soluble peptide content and antioxidant activity found in HSM and FHSM, (**b**) Trypsin inhibitor and Urease levels in HSM and FHSM.

Bacillus were the main producers of extracellular proteases, and solid-state fermentation was confirmed to be an efficient method of increased protease production in *B. subtilis* [32]. During fermentation, proteases secreted by *B. subtilis* partially digested macromolecular proteins in the HSM, resulting in an increase in the proportion of soluble polypeptides of small molecules from 6.07% to 15.15%. Bi et al. demonstrated similar results when using soybean meal fermentation to produce plasmin, with large and medium protein molecules becoming lower and the small protein levels increasing [33]. *B. subtilis* secretes abundant proteases that are able to enzymatically cleave proteins in HSM, and a small molecule nitrogen source is in turn more conducive to nutrient uptake by cells. Additionally, the antioxidant activities of soluble polypeptides before and after fermentation were determined. The hydroxyl radical removal ability was improved from 9.20% to 14.85%, and the DPPH radical removal ability was improved from 22.02% to 32.96% after fermentation. Proteolysates from hemp seeds have been shown to exhibit in vitro antioxidant activity [34]. The polypeptides produced during solid-state fermentation of HSM have high potential for application as antioxidants.

3.6. Anti-Nutritional Factors in HSM

The anti-nutritional component in hemp seeds is the reason why its application is limited [35]. Protease inhibitors and urease antinutritional activity cause growth inhibition and pancreatic hypertrophy [36,37]. Many cultivated legumes and plant seeds contain protease inhibitors, and hemp seed meal is alike in this regard. It has been shown that the content of anti-nutritional factors in plant seeds after fermentation decreased [38]. In this study, inoculation of *B. subtilis* 13,932 on HSM reduced trypsin inhibitor levels by 42.6% and urease by 73.6% after fermentation. Fermentation effectively reduced the anti-nutritional content of HSM, which is beneficial to the application of HSM for use in food products.

3.7. HSM Sensory Analysis

Objective measures of sensory analysis are appearance, smell, unique flavor, and textural characteristics [39]. Sensory analysis techniques are highly significant in the development of novel foods. The radar plot below visually exhibits the scoring across all aspects (Figure 4). The findings indicated that HSM after fermentation was evaluated higher for stringiness and texture. After fermentation, HSM was altered from faint yellow to pyro yellow. HSM has a

unique flavor after fermentation, which improved the smell of fermentation to some extent. In taste, it was slightly bitter, which is unique to NK fermented foods [18].

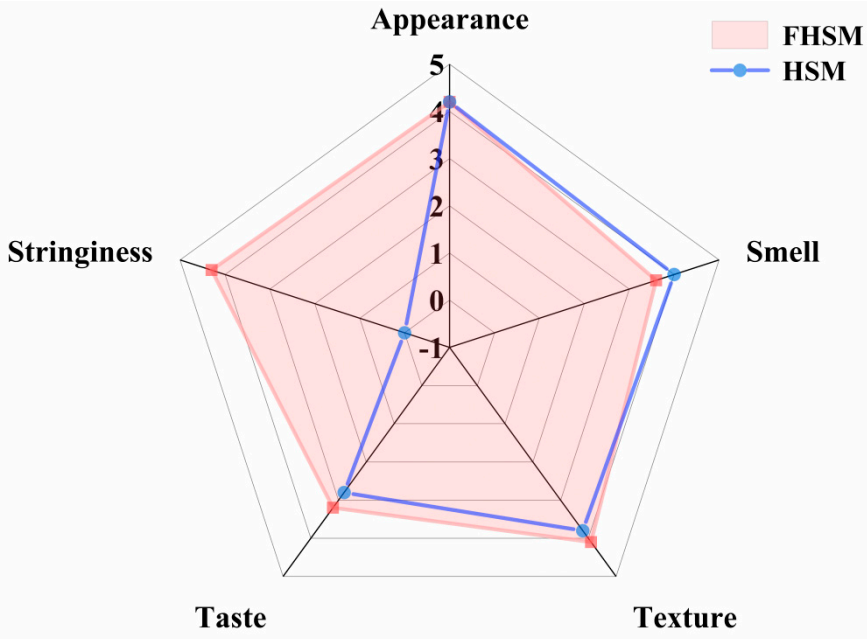

Figure 4. Sensory analysis of FHSM and HSM.

In this study, solid-state fermentation of HSM was used to obtain highly active NK, which, after isolation and purification, is expected to provide a highly active oral formulation of NK for the treatment of thrombotic diseases [40]. In addition, solid-state fermentation of HSM to produce NK resulted in the production of peptides with antioxidant activity, and the low-cost substrate itself became valuable. Moreover, *B. subtilis* is biosafe, and the fermented HSM as a whole could have promising applications as a prebiotic in feed and pet food. This research provides a new way of thinking about the use of process wastes like HSM, and it is expected that in the future, more cheap wastes will be used to produce highly active and valuable enzymes.

4. Conclusions

In this study, the major components of HSM (moisture, protein, fat, starch, and dietary fiber) were determined. The optimal fermentation conditions were determined through optimization of the production of NK in solid-state fermentation of HSM using OFAT, PB design, and BBD. Soluble polypeptides with antioxidant activity were increased in fermented HSM compared to unfermented HSM. Moreover, the anti-nutritional factors found in HSM were effectively reduced after fermentation. Sensory analysis demonstrated that HSM after fermentation was acceptable. In summary, fermenting HSM is a feasible method for production of NK with high nutritional value.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9050469/s1, Table S1: PB Design results; Table S2: BBD results.

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