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Optimize the fermentation medium of the extruded soy sauce residue by Plackett-Burman combined with Box-behnken response surface method

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Abstract: *Selected the pretreated soy sauce residue by extrusion as the experimental material, the ratio test of Aspergillus niger, Bacillus subtilis, Candida utilis and Geotrichum candidum was carried out based on the* $L_9(3^4)$ *orthogonal test design; the composition of the medium was optimized by the Plackett-Burman test, the steepest climbing test and the Box-behnken response surface test with the true protein and crude fiber content as the inspection indicators, the optimal stains ratio was determined as Aspergillus niger: Bacillus subtilis: Candida utilis: Geotrichum candidum=3:2:1:3, the optimal medium composition was glucose of 1%, sucrose of 1%, urea of 1%, ammonium chloride of 1.8%, magnesium sulfate of 0%, disodium hydrogen phosphate of 0%, material/water ratio of 1:1.4 and bran of 22.72%. Under the above fermentation conditions, the true protein and crude fiber content in the fermented product were 31.15% and 18.53%, respectively. Compared with the extruded soy sauce residue, the true protein content was increased by 17.11% and the crude fiber content was reduced by 19.78%. This research provided data support for the production of protein feed by fermenting extruded soy sauce residue, and provided a potential idea for the development of the soy sauce industry.*
Key words: *Extruded soy sauce residue; Mixed fermentation; Plackett-Burman experimental design; Medium optimization*

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I. Introduction

Soy sauce is a traditional condiment in China, which occupies the largest market proportion of condiment industry and plays an irreplaceable role. In recent years, it has maintained a steady growth in market demand. Sauce residue is the residue left after the sauce mash has been extracted. According to the database report of China Academy of Commerce in 2019, the output of soy sauce in China reached 7.08 million tons in 2020. If 0.67 kg of fresh sauce residue was produced for every 1 kg of soy sauce, there will be about 4 million tons of fresh sauce residue, and the output of sauce residue increased year by year [1,2]. Soy sauce residue was rich in nutrients, such as about 20% - 30% crude protein, 20% - 30% crude fiber, 8% - 10% crude fat and rich soybean isoflavones [3,4]. It was a potential cheap biomass resource as fermentation substrate to produce protein feed, which not only improved the economic development of soy sauce industry and the utilization rate of soy sauce residue, but also reduced the production cost of fermentation products and reduced the environmental pollution.

At present, the utilization of soy sauce residue mainly included extraction of oil and antioxidant substances [5-8], microbial fermentation with *Streptomycin* to produce biogas [9,10], microbial fermentation to produce feed [4,11,12], preparation of immunoactivity peptide [13] and edible film [14] and so on. However, the utilization of soy sauce residue was relatively simple, and it cannot fundamentally solve the problems of the large output of soy sauce residue, which resulted in the serious waste of resources and environmental pollution. Extrusion technology improved the structure and composition of raw materials, and enhanced the nutritional value and utilization rate of raw materials. Therefore, extrusion technology was used to pretreat the soy sauce residue, and then to prepare feed by the multi strain fermentation. However, the mixed fermentation of extruded soy sauce residue had not been reported yet.

The aim of this study was to optimize the proportion of mixed strains and the composition of culture medium of the fermenting extruded soy sauce residue. Based on $L_9(3^4)$ orthogonal test design, the proportion of *Aspergillus niger*, *Bacillus subtilis*, *Candida utilis* and *Geotrichum candidum* was optimized; The best medium composition was determined by Plackett-Burman test, steepest climbing test and Box-Behnken response surface test with the true protein and crude fiber of fermentation products as the indexes. Therefore, this study provided

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an important reference for industrial utilization and fermentation fodder.

II. Materials and Methods

2.1 Materials and reagents

Soy sauce residue (the raw material is 65% soybean meal and 35% wheat, adding 17% salt to get soy sauce by circulating pouring method): Shandong Yutu Food Co., Ltd; *Aspergillus niger* 3.324 and *Bacillus subtilis* were provided by Yiyuan Kangyuan Biotechnology Co., Ltd; *Geotrichum candidum* 1315 and *Candida utilis* 1314 were purchased from Agricultural Culture Collection of China; The reagents used in the test were analytical pure: Sinopharm Chemical Reagent Co., Ltd.

Aspergillus niger 3.324 was activated with modified Martin medium for 24 h; *Bacillus subtilis* was activated with LB medium for 24 h; *Candida utilis* 1314 and *Geotrichum candidum* 1315 were activated with YPD medium for 24 h.

2.2 Main instruments and equipments

Single screw extruder, Shandong University of Science and Technology Agricultural products processing and storage laboratory; K9860 Automatic Kjeldahl nitrogen analyzer, Jinan Haineng Instrument Co., Ltd.

2.3 Test method

2.3.1 Preparation of extrusion sauce residue

The results showed that the optimum extrusion conditions were as follows: extrusion temperature of 103℃, moisture content of 34% and screw speed of 98 R/min.

2.3.2 Determination of true protein content

For the determination of true protein content, it referred to the method of Li et al. [15].

2.3.3 determination of crude fiber content

For the determination of crude fiber content, it referred to Al-sheraji [16] and other methods.

2.3.4 mixed strain ratio test

Aspergillus niger secreted cellulase to degrade cellulose, *Bacillus subtilis* secreted protease to degrade protein, *Candida utilis* and *Geotrichum candidum* were enriched proteins. According to the previous literature reading and the role of different strains, it can be determined that multi strain co-fermentation is better than single strain or double strain fermentation. Therefore, based on the orthogonal test table of $L_9(3^4)$, the proportion test of *Aspergillus niger*, *Bacillus subtilis*, *Candida utilis* and *Geotrichum candidum* was carried out. The specific test arrangement was shown in Table 1. Different strains were added into the solid medium containing 15 g extruded sauce residue, the ratio of material to water was 1:1.5, and the inoculation amount was 15%. After shaking well, the triangular flask was put into 30℃ incubator for 4 days, and then dried after fermentation. The true protein and crude fiber content were determined after fermentation.

Table 1 Designed ratio test of different strains based on $L_9(3^4)$ orthogonal table

Run	*Aspergillus niger*	*Bacillus subtilis*	*Candida utilis*	*Geotrichum candidum*
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

2.3.5 Pleckt-Berman experimental design

Microbial growth was inseparable from nutrients such as carbon source, nitrogen source, inorganic salt and water. Therefore, eight factors including carbon source (Glucose, sucrose), nitrogen source (Urea, ammonium chloride), inorganic salt (Magnesium sulfate, Disodium hydrogen phosphate), water and bran were investigated to determine the significant factors affecting microbial growth. The specific test factors and levels were shown in Table 2. The fermentation conditions were 2.3.4, and the contents of true protein and crude fiber were determined after fermentation.

Table 2 Plackett-Burman test design factors and levels

level	*A* Glucose /%	*B* Sucrose /%	*C* Urea /%	*D* Ammonium chloride /%	*E* Magnesium sulphate /%	*F* Disodium hydrogen phosphate/ /%	*G* Material water ratio	*H* Bran /%
-1	1	1	1	1	0	0	1:1	10
1	2	2	2	2	1	1	1:1.5	30

2.3.6 Steepest climbing test

Based on the Plackett Burman test, the best region of significant factors was determined by climbing test, and the best medium composition was further determined. The value of positive effect increased gradually, and the value of negative effect decreased gradually. The best test step was designed to determine the accuracy of the test results.

2.3.7 Box-Behnken response surface test

According to Plackett Burman design and climbing test, response surface analysis of 15 test points with 3 factors and 3 levels was designed. The results were analyzed by design expert software, and the optimal composition of fermentation medium was predicted according to the regression equation. The optimal fermentation conditions were 1.3.4, and the contents of true protein and crude fiber were determined after fermentation.

2.4 Data statistics and analysis

Design expert 8.0.6 was used for data processing and analysis, including regression analysis, quadratic polynomial regression equation, analysis of variance and response surface analysis; Use origin Pro 9.1 software to draw. All tests were repeated three times.

III. Results and analysis

3.1 Experiment on the proportion of mixed strains

It was seen from Fig 1 that *Aspergillus niger*: *Bacillus subtilis*: *Candida utilis*: *Geotrichum candidum*=3:2:1:3, the true protein content reached the maximum value of 27.31%, and the crude fiber content reached the minimum value of 20.12%. Under these conditions, *Aspergillus niger* had the best cellulose decomposition effect, and *Candida utilis* and *Geotrichum candidum* had the largest protein accumulation. Therefore, *Aspergillus niger*: *Bacillus subtilis*: *Candida utilis*: *Geotrichum candidum*=3:2:1:3 was selected to optimize the medium composition.

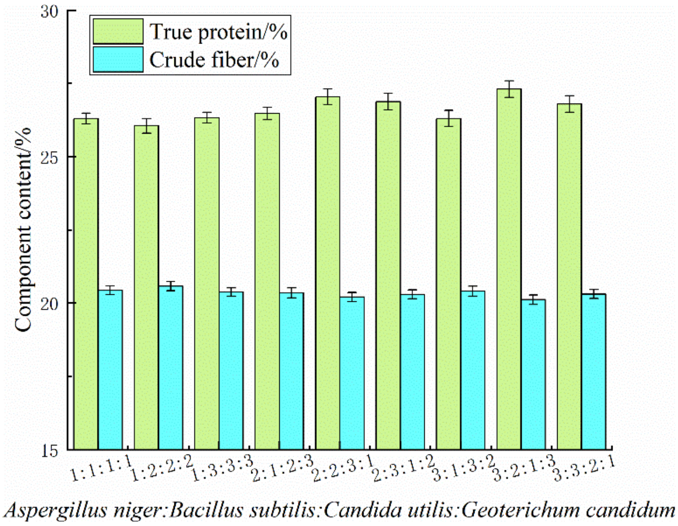

Fig 1 Effect of different strain ratios on true protein and crude fiber

3.2 Analysis results of Plackett-Burman test

Plackett-Burman test was used to screen out the factors that had significant effect on true protein and crude fiber, so as to further optimize the composition of culture medium and determine the best composition of culture medium. According to the design expert software, the number of experiments was 12, and each group was repeated three times. The real protein and crude fiber were taken as the test results. The arrangement and results of the experiment were shown in Table 3.

Table 3 Plackett-Burman design with experimental results

Run	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	True protein /%	Crude fiber /%
1	1	-1	1	-1	1	1	-1	-1	27.764	19.56
2	-1	-1	1	-1	1	-1	1	1	25.177	20.61
3	-1	-1	-1	1	-1	1	1	-1	24.819	20.87
4	-1	1	1	1	1	1	-1	-1	26.961	19.85
5	1	1	1	1	-1	-1	1	-1	25.466	20.47
6	-1	1	1	-1	-1	1	1	1	25.328	20.53
7	-1	1	-1	1	1	-1	-1	1	26.51	20.02
8	1	-1	-1	1	1	1	1	1	23.861	21.18
9	-1	-1	-1	-1	-1	-1	-1	-1	27.566	19.71
10	1	1	-1	-1	-1	1	-1	1	26.102	20.11
11	1	-1	1	1	-1	-1	-1	1	25.784	20.32
12	1	1	-1	-1	1	-1	1	-1	26.007	20.15

Table 4 Analysis of variance of true protein and crude fiber regression model

Source	F_1 value	P_1 value	F_2 value	P_2 value
Constant	15.63	0.0226	25.64	0.0111
A	1.43	0.3178	0.28	0.6354
B	1.48	0.3102	8.67	0.0603
C	1.97	0.2554	3.39	0.1630
D	15.56	0.0291	28.77	0.0127
E	1.11	0.3689	2.83	0.1910
F	2.11	0.2419	4.65	0.1200
G	75.82	0.0032	124.27	0.0015
H	25.54	0.0149	32.25	0.0108
	R_1^2=09766		R_2^2=0.9856	

Note: F_1, P_1, R_1 as true proteins, F_2, P_2, R_2 as crude fibers.

3.3 Analysis results of steepest climb test

According to the variance analysis of Plackett-Burman regression model, it was concluded that the significant influence factors of ammonium chloride, material water ratio and bran were all positive effects. The steepest climbing test was carried out for these three factors, and the specific test arrangement was shown in Table 5. The other components were glucose 1%, sucrose 1%, urea 1%, magnesium sulfate 0% and disodium hydrogen phosphate 0%.

It was seen from Table 5 that when ammonium chloride and wheat bran were added at 1.6% and 22% respectively, and the ratio of material to water was 1:1.6, the true protein content reached the maximum and the crude fiber content reached the minimum, which was taken as the central point of Box-Behnken response surface test to optimize the composition of the medium.

Table 5 Steepest ascent design with experimental results

Run	*A* Ammonium chloride（%）	*B* Material water ratio	*C* bran（%）	True protein /%	Crude fiber /%
1	1.2	1:1.2	14	28.773	19.41
2	1.4	1:1.4	18	28.451	19.64
3	1.6	1:1.6	22	28.798	19.32
4	1.8	1:1.8	26	28.285	19.71
5	2.0	1:2.0	30	27.101	19.88
6	2.2	1:2.2	34	26.653	19.91
7	2.4	1:2.4	38	26.484	19.97
8	2.6	1:2.6	42	26.186	20.04

3.4 Analysis results of Box-Behnken response surface test

Box-Behnken response surface test was carried out for the best A, B and C obtained from the steepest climbing test. The specific test arrangement and results were shown in Table 6. The results of ANOVA of true protein and crude fiber were shown in Table 7. The experimental results were fitted and analyzed by design expert software, and the quadratic regression equations were obtained as follows:

$Y_{True\ protein}$=30.45+0.49A+0.098B+0.21C-0.25AB+0.095AC-0.005BC+0.09A^2+0.015B^2-0.3C^2,
$Y_{Crude\ fiber}$=18.52+0.14A+0.24B-0.017C+0.15AB-0.06AC-0.055BC+0.088A^2+0.083B^2+0.038C^2.

From the analysis of variance table, it was seen that the P values of true protein regression equation model (P_1 = 0.0386) and crude fiber regression equation model (P_2 = 0.0005) were less than 0.05, which indicated that the regression model had significant differences, and the P values of mismatch items were greater than 0.05, which indicated that the regression equation was reasonable and feasible. From R_1^2 = 0.9071, $R_{1\ adj}^2$ = 0.7398, R_2^2 = 0.9850, $R_{2\ adj}^2$ = 0.9581, it was seen that the test results are close to the predicted values, so the reliability and precision of the test were high. It was seen from the table that A had a very significant effect on the true protein; A, B and AB had significant effect on crude fiber content, A^2 and B^2 had significant effect on crude fiber content, other items had no significant effect on true protein and crude fiber. According to the F value, the influence of various factors on the true protein in the experimental range was A > C > B; the effect on crude fiber was B > A > C. In conclusion, the established model was consistent with the experimental data, so the influence of various factors on true protein and crude fiber can be predicted and analyzed.

Table 6 Test results of Box-Behnken response surface

Run	*A*	*B*	*C*	True protein /%	Crude fiber /%
1	1	-1	0	31.43	18.45
2	-1	1	0	30.17	18.64
3	-1	0	-1	29.85	18.51
4	1	1	0	30.91	19.26
5	0	-1	-1	29.65	18.37
6	1	0	1	30.82	18.67
7	0	1	-1	30.08	18.91
8	-1	0	1	29.91	18.55
9	0	0	0	30.46	18.55
10	1	0	-1	30.38	18.87
11	0	0	0	30.36	18.52
12	-1	-1	0	29.71	18.43
13	0	1	1	30.67	18.81
14	0	0	0	30.53	18.50
15	0	-1	1	30.26	18.49

Table 7 Analysis of variance of true protein and crude fiber regression model

Source of variance	F_1 值	P_1 值	F_2 值	P_2 值
Model	5.42	0.0386	36.54	0.0005
A	30.95	0.0026	66.91	0.0004
B	1.24	0.3165	188.53	< 0.0001
C	5.88	0.0597	1.05	0.3534
AB	3.91	0.1050	38.41	0.0016
AC	0.59	0.4779	6.15	0.0559
BC	0.00	0.9694	5.16	0.0722
A^2	0.49	0.5164	12.29	0.0172
B^2	0.01	0.9119	10.94	0.0213
C^2	5.41	0.0676	2.32	0.1886
Misfit term	13.36	0.0704	5.50	0.1577
	R^2=0.9071	R^2_{Adj}=0.7398	R^2=0.9850	R^2_{Adj}=0.9581

It was seen from table 7 that AB interaction term had a very significant impact on crude fiber. Response surface analysis diagram was drawn by software, as shown in Fig 2. It was seen from the figure that with the increase of the content of ammonium chloride, the content of crude fiber decreased first and then increased while the ratio of material to water remained unchanged. When the content of ammonium chloride reached 1.8%, the content of crude fiber reached the minimum. This may be because the appropriate amount of ammonium chloride was conducive to the growth and reproduction of microorganisms which secreted a lot of cellulase to degrade cellulose, and decreased the content of crude fiber; If the content of ammonium chloride continued to increase, the ion concentration in the solid medium was increased, the reverse osmosis pressure was increased, the growth of microorganisms or the synthesis of cellulase was inhibited, and the activity of cellulase was reduced, thus the degradation ability of cellulose was weakened, resulting in the increase of crude fiber content [17, 18]. If the content of ammonium chloride remained unchanged, the content of crude fiber decreased and then increased with the increase of the ratio of material to water. When the ratio of material to water was 1:1.4, the content of crude fiber was reached the minimum. This may be because the appropriate amount of water content made the solid medium had good fluffy, improved the gas exchange capacity, enhanced the stability of extracellular enzymes and the solubility of nutrients to provide a suitable environment for the growth of microorganisms and promote the synthesis of cellulase, so the crude fiber content was decreased [19-21]. When the water content was high, the solid medium showed sticky phenomenon, resulting in poor air permeability and low dissolved oxygen content of the solid medium, which was not conducive to the growth and reproduction of microorganisms, resulting in the increase of crude fiber content [22-24].

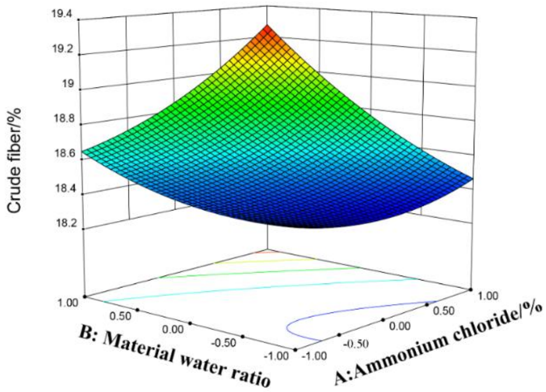

Fig 2 Response surface diagram of the influence of ammonium chloride and material/water ratio on the true protein content

3.5 Verification test

According to the quadratic regression equation, the optimal combination of factors affecting the true protein and crude fiber in fermented extruded soy sauce residue was glucose 1%, sucrose 1%, urea 1%, ammonium chloride 1.8%, magnesium sulfate 0%, disodium hydrogen phosphate 0%, material water ratio 1:1.4 and bran 22.72%. At this time, the theoretical maximum valued of true protein and crude fiber in fermented extruded soy sauce residue were 31.27% and 18.45%, respectively.

Through three parallel experiments, the real protein and crude fiber in the fermented extruded soy sauce residue were 31.15% and 18.53% respectively, and the error was less than 5%. Therefore, the quadratic regression model was reasonable and feasible.

IV. Conclusion

In this study, the extrusion pretreatment of soy sauce residue fermentation experiment, a variety of bacteria co-fermentation and medium composition were explored. Based on $L_9(3^4)$ orthogonal design, the optimal ratio of *Aspergillus niger*: *Bacillus subtilis*: *Candida utilis*: *Geotrichum candidum* was 3:2:1:3; Under the optimal ratio, Plackett-Burman test, the steepest ascent test and Box-Behnken response surface test was used to determine the optimal medium group as follows: glucose 1%, sucrose 1%, urea 1%, ammonium chloride 1.8%, magnesium sulfate 0%, disodium hydrogen phosphate 0%, material water ratio 1:1.4 and bran 22.72%. At this time, the content of true protein and crude fiber in the fermentation product were 31.15% and 18.53%, respectively, compared with the unfermented soy sauce residue, the true protein content increased by 17.11%, and the crude fiber content decreased by 19.78%, which laid a foundation for the subsequent fermentation of extruded soy sauce residue to prepare protein feed.

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