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Original paper

Optimization of Fermentation Process for Improving Soy Isoflavones Aglycone Content in Bean Dregs by Lactobacillus plantarum PL70a

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Abstract Aglycone-type soy isoflavones have higher biological activity than glycoside-type soy isoflavones. Bean dregs are rich in glycoside-type soy isoflavones. In order to improve the biological activity of soy isoflavones in bean dregs, the *Lactobacillus plantarum* PL70a was screened and the fermentation process of converting glycoside-type soy isoflavones into aglycone-type in bean dregs was optimized by single-factor experiments and the response surface methodology. The optimal fermentation process was as follows: $(NH_4)_2SO_4$ was added in an amount of 0.17%, glucoamylase was added in an amount of 0.87%, inoculation amount was 17%, sucrose was added in an amount of 1.3%, and fermentation time was 3 days. Under this process, the content of aglycone-type soy isoflavones in bean dregs significantly increased. Trypsin inhibitors and antigen proteins were almost removed. The fermentation process provides a good reference for the low-cost processing of high-quality soy isoflavone aglycone food.

Keywords Soy isoflavones, Bean dregs, *Lactobacillus plantarum*, Fermentation, Response surface methodology

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Introduction

Soy isoflavones (SI) are a kind of natural active ingredient isolated from soybeans. Forms of SI mainly include glycoside-type soy isoflavones (GTSI) (daidzin, genistin, and glycitin) and aglycone-type soy isoflavones (ATSI) (daidzein, genistein, and glycitein) (CHADHA & al. [1]). The GTSI content is 50-90% of the total SI content (CAGNO & al. [2]). A large number of studies and animal experiments have shown that SI are phytoestrogens and have a variety of physiological and pharmacological functions, such as antioxidant, antibacterial, anti-cancer, improving the immune function, preventing osteoporosis and leukemia, etc. (BROUNS [3]; KOBAYASHI & al. [4]; KUCUK [5]; XU & al. [6]). The biological effects of SI are strongly influenced by their chemical structure (ANDRADE & al. [7]). GTSI cannot pass through the small intestinal wall, and needs to be hydrolyzed into aglycons by enzymes secreted by microorganisms in the intestine to be absorbed (XU & al. [8]).

Bean dregs are the by-products in the processing of soybean products such as tofu and soy milk (RU & al. [9]). Bean dregs are rich in nutrients and contain a large amount of SI (ZHU & al. [10]), mainly GTSI. They are a kind of high-quality food to supplement SI. Various reports have indicated that the biological effects of SI are not due to GTSI but mainly to ATSI, such as daidzein and genistein (KAWAKAMI & al. [11]; KUO & al. [12]). ATSI have higher biological activity than GTSI and degradation of GTSI into ATSI can significantly improve the bioavailability of SI (AHN-JARVIS & al. [13]).The research and development of SI in bean dregs can effectively improve the bioavailability of bean dregs and have a very broad application prospect.

At present, the methods for converting GTSI to ATSI mainly include enzymatic hydrolysis, acid hydrolysis and so on (KLEJDUS & [14]). Acid hydrolysis methods have many disadvantages including low conversion rate, acute reaction conditions and being harmful to human health. The conversion efficiency of enzymatic hydrolysis method is high (ANISH & al. [15]). However, the price of enzyme is very high, so the production cost of enzymatic hydrolysis method is also high. Neither method is suitable for large-scale processing applications. In recent years, studies have shown that a variety of microorganisms can secrete the enzymes needed to convert GTSI into ATSI, such as *Bacillus subtilis*, *lactobacillus plantarum*, *Aspergillus Niger* and so on (CAO & al. [16]; LEE & al. [17]; ZHU & al. [18]). In addition, microbial fermentation has the advantages of no pollution, high conversion efficiency, and low cost. Therefore, microbial fermentation has a wide range of applications in the conversion studies of ATSI.

In this study, bean dregs were used as raw materials. Through the screening of dominant fermentation strains, single-factor experiments, and response surface methodology, the optimal fermentation process for increasing the content of ATSI was obtained. This fermentation process provides a good reference for the low-cost processing of high-quality soy isoflavone aglycone food, which has great economic benefits and application value.

Materials and Methods

Materials and reagents

The bean dregs were purchased from a bean processing factory in Zhenjiang City (Jiangsu, China) with a water content of about 80%. All solvents and chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glucoamylase for the experiment was food grade with an activity of about 150000 u/g. The purity of solvents and chemicals used in the HPLC were HPLC grade, and the others were of analytical reagent grade.

Microorganisms

The *Candida utilis* CGMCC 2.1180, *Saccharomyces cerevisiae* CGMCC 2.1527, *Bacillus natto* CGMCC 1.1086, *Bacillus licheniformis* CGMCC1.813, and *Lactobacillus acidophilus* CGMCC 1.2467 were provided by the China General Microorganisms Collection and Management Center. The *Lactobacillus plantarum* PL70a, *Aspergillus niger* CMCC (F) 98003, and *Aspergillus niger* ATCC 16404 were screened and preserved by our laboratory.

Screening of dominant fermentation strains

200 g bean dregs were used as the solid fermentation medium. Other independent variables remained unchanged. The above eight strains were respectively inoculated into the solid fermentation medium, and the inoculation amount of each strain was 18%(v/w). Then, the medium was placed in a sealed fermentation bag and fermented at 30°C. The content of ATSI was measured on the eighth day of fermentation, and the optimal fermentation strain was selected.

Single-factor experiments

Other factors remained unchanged, and different carbon sources (1% sucrose, glucose, soluble starch, bran, brown sugar, w/w), fermentation time (1, 2, 3, 5, 7, 9, 11days), sucrose addition amount (0.4%, 0.7%, 1%, 1.3%, 2%, 3%, 4%, w/w), inoculation amount of *Lactobacillus plantarum* PL70a (1%, 5%, 9%, 13%, 17%, 21%, v/w), glucoamylase addition amount (0.1%, 0.5%, 0.9%, 1.3%, 1.7%, 2.1%, w/w), $(NH_4)_2SO_4$ addition amount (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.6%, w/w) were selected successively for optimization experiments. Other fermentation processes were the same as above.

Response surface methodology (RSM)

Based on the results of single-factor experiments, the $(NH_4)_2SO_4$ addition amount, glucoamylase addition amount, and inoculation amount were selected as the factors to investigate, and the the increase rate of ATSI was taken as the index. Box-Behnken Design (BBD) was used to design the optimization experiments. Experimental factors and levels are shown in Table 1.

Determination of SI

Extraction and determination of SI referred to ANDRADE & al. [7]. 1.00 mg Daidzin, genistin, daidzein, genistein and glycitein standard were respectively taken and diluted to 5 μg/mL, 10μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, and 60 μg/mL with 80%(v/v) ethanol. The samples

were filtered through 0.45 μm filters for HPLC analysis, and standard curves were drawn.

2.00 g dried sample was dissolved by adding 30 mL 80%(v/v) ethanol. It was left at 50℃ for 2h, and then centrifuged at 7580×g for 10 min. The supernatant was taken and filtered through a 0.45 μm filter for HPLC analysis. Chromatographic conditions: mobile phase: methanol : 1%(v/v) acetic acid = 70:30; chromatographic column: C18 column (Prontosil, 120-5-C18-ace-EPS, 5.0 μm); detection wavelength: 254 nm; flow rate: 1 mL/min; column temperature: normal temperature. The increase rate of ATSI was calculated using the equation (1). Increase rate of ATSI (%) $= \frac{X_2 - X_1}{X_1}$ (1)

X_1 is the content of ATSI before fermentation, including daidzein, genistein and glycitein. X_2 is the content of ATSI after fermentation, including daidzein, genistein and glycitein.

Determination of trypsin inhibitors

Determination of trypsin inhibitors was based on the method reported by HE & al. [19]. 1.00 g sample was mixed with 30 mL 0.01 mol/L NaOH and was shaken at 30°C for 3 h.Then,it was centrifuged at 1000×g for 10 min. The supernatant was taken for experiments.The removal rate of trypsin inhibitors was calculated using the equation (2).

Removal rate of trypsin inhibitors (%) $= \frac{A_1 - A_2}{A_1}$ (2)

A_1 is the content of trypsin inhibitors before fermentation. A_2 is the content of trypsin inhibitors after fermentation.

Determination of antigen proteins

The extraction of antigen proteins and SDS-PAGE referred to MENG & al. [20] and LAEMMLI [21]. 1.00 g dried sample was mixed with 30 mL 0.03 mol/L Tris-Hcl solution, soaked at room temperature for 1 h, centrifuged at 16520×g for 10 min. The supernatant was used for SDS-PAGE.

Statistical analysis

All the experiments were performed in triplicate, and the data were expressed as mean ± SD. Response surface design and data analysis were performed using Design-Expert 8.0.6, and the other data were analyzed using the Origin Pro8 software.

Table 1 Optimization of fermentation process factors and levels by RSM

Level	A $(NH_4)_2SO_4$ addition amount (%)	B Glucoamylase addition amount (%)	C Inoculation amount (%)
-1	0.1	0.5	9
0	0.2	0.9	13
1	0.3	1.3	17

Results and Discussion

Standard curves of five kinds of SI

Standard curves of five kinds of SI in bean dregs are shown in Table 2. The all standard curves had good linearity, and R^2 was greater than 0.99, which can be used for the determination of SI.

Screening of dominant fermentation strains

As shown in Figure 1, each experimental group was compared with the control group (raw material without added strain). In addition to the negative effects of *Saccharomyces cerevisiae* and *Candida utilis*, other strains have the ability to convert GTSI to ATSI. Among them, the *Lactobacillus plantarum* PL70a had the best conversion effect, so the *Lactobacillus plantarum* PL70a was selected as the optimal fermentation strain.

The effect of different carbon sources

As shown in Figure 2, when the carbon source was sucrose, the ATSI had the highest increase rate.The sucrose was more suitable for the growth of *Lactobacillus plantarum* PL70a than other carbon sources, so the sucrose was selected.

The effect of fermentation time

As shown in Figure 3, in the early stage of fermentation, the content of ATSI increased rapidly with the increase of fermentation time. Due to sufficient nutrients in the solid medium, strains grew rapidly and secreted a large amount of enzymes, and the conversion efficiency of ATSI improved continuously. After fermentation for 3 days, it began to moderate and showed a slow growth trend. In the later stage of fermentation, nutrition is insufficient and the strain gradually aged or died (DELGADO & al. [22]), so the conversion efficiency of ATSI was reduced. Therefore, the fermentation time was selected to be 3 days.

The effect of sucrose addition amount

As can be seen from the previous studies, sucrose was a suitable carbon source for the fermentation strains used in this experiment. The sucrose actes as an additional carbon source for bean dregs fermentation medium to provide energy for the growth of microorganisms and better promoted the growth and reproduction of the strains (GOKSUNGUR & al. [23]). The enzyme production capacity was further improved, which promoted the conversion of ATSI in the bean dregs. However, too much sucrose will not only cause waste of resources, but also

cause nutritional imbalance and excessive osmotic pressure of the medium, which will cause the strain to lose water and the cytoplasmic wall to separate and adversely affect the growth of microorganisms. As shown in Figure 4, When the addition amount of sucrose was 1.3%, the increase rate of ATSI reached the maximum (63.12%). Therefore, the optimal addition amount of sucrose was 1.3%.

The effect of inoculation amount

As shown in Figure 5, the inoculation amount had a great impact on the increase rate of ATSI. As the inoculation amount was 13%, the increase rate of ATSI reached the maximum value. When the inoculation amount was too small, the strain took a long time to grow and reproduce, and the fermentation process was slow. When the inoculation amount was too large, the excessive growth of the fermentation strain caused the nutrients in the solid fermentation medium was quickly consumed, and the pH was lowered too fast,thereby inhibiting the growth of the strain. This experimental results were consistent with those of CHAMPAGNE & al. [24] who reported that too large or too small inoculation was not conducive to the conversion of GTSI into ATSI. Therefore, the optimal inoculation amount was 13%.

The effect of glucoamylase addition amount

The study has reported that the addition of some enzyme preparations such as glucoamylase, β-glucosidase, β-glucanase could hydrolyze GTSI into ATSI (CAO & al. [16]). This study found that the glucoamylase had a great impact on the increase rate of ATSI. As shown in Figure 6, the appropriate amount of glucoamylase was beneficial to the increase rate of ATSI. This might be that glucoamylase could hydrolyze the starch in the fermentation medium to glucose. The previous carbon source experiments showed that glucose was more conducive to the conversion of ATSI than starch. When the addition amount of glucoamylase was 0.9%, the increase rate of ATSI reached the maximum (62.19%). When it was higher than 0.9%, it showed a downward trend. This was due to the fact that the excess glucoamylase inhibited the growth and reproduction of *Lactobacillus plantarum* PL70a, and the fermented strain and the added enzyme were not able to be fermented synergistically. It was also possible that the enzyme preparation contained some unidentified substance which inhibited the growth of *Lactobacillus plantarum* PL70a. Therefore, 0.9% was selected as the glucoamylase addition.

The effect of $(NH_4)_2SO_4$ addition amount

An appropriate amount of nitrogen source added into the fermentation medium is beneficial to the growth of the fermentation strain (LEE & al. [25]). As shown in Figure 7, when the $(NH_4)_2SO_4$ addition amount increased, the increase rate of ATSI kept increasing. As the $(NH_4)_2SO_4$ addition amount was 0.2%, the increase rate of the ATSI reached the maximum (60.16%). However, when the $(NH_4)_2SO_4$ addition amount was too high, the fermentation environment was not conducive to the growth of the fermentation strain due to the large accumulation of ammonium nitrogen, and the conversion efficiency of ATSI was lowered. Therefore, the optimal $(NH_4)_2SO_4$ addition amount was 0.2%.

RSM results and analysis

According to the single-factor experiments, the conversion of ATSI was mainly affected by the corresponding enzyme, and the amount of inoculation amount and the glucoamylase addition amount directly affected the amount of enzyme. The addition of a small amount of $(NH_4)_2SO_4$ promoted the conversion of ATSI, but a slight increase had a significant inhibitory effect. The $(NH_4)_2SO_4$ addition amount, glucoamylase addition amount, and inoculation amount need further optimization. Therefore, they were selected as independent variables. The increase rate of ATSI was used as the response value, and the experiment of three factors and three levels was designed. The experimental schemes and results are shown in Table 3.

The quadratic multiple regression equation of A ($(NH_4)_2SO_4$ addition amount), B (glucoamylase addition amount), and C (inoculation amount) was:

$$Y \text{ (Increase rate of ATSI)} = 67.08-2.36A-0.29B+5.65C-2.29AB-3.43AC-1.14BC-9.52A^2-4.98B^2-1.53C^2 \quad (3)$$

The results of the variance analysis of the regression equation are shown in Table 4. The regression model for the increase rate of ATSI was extremely significant ($p < 0.0001$), indicating that there were significant differences in the increase rate of ATSI under different conditions. The lack of fit was not significant ($0.1891 > 0.05$), indicating that the predicted value of the model was well fitted to the actual value and the equation can describe the relationship between different conditions and the increase rate of ATSI well. The regression coefficient of the model was $R^2=0.9880$ and $AdjR^2=0.9725$, which indicated that the model fitted well with the experiment and the linear relationship between the independent variable and the response value was significant (PRAKASH & al. [26]). Adeq precision ($20.786 > 4$) indicated that the equation model can be used to predict experimental data well. The analysis showed that the model was suitable.

According to the analysis results, the order of influence of the selected factors on the increase rate of ATSI was as follows: inoculation amount (C) > $(NH_4)_2SO_4$ addition amount (A) > glucoamylase addition amount (B). The effects of factors A, C, AB, AC, A^2, and B^2 on the increase rate of ATSI were extremely significant ($p < 0.01$); the effects of factor C^2 on the increase rate of ATSI were significant ($p < 0.05$). The effects of factors B and BC on the increase rate of ATSI were not significant ($p > 0.05$).

Response surface plots are shown in Figure 8. The response surface plots reflected the significance of each factor. The steeper the 3D response surface plot was, the greater the influence of this factor on the response values was (WANG & al. [27]). The contour plot showed the significance of the interaction between the two variables. The more the contour lines tend to ellipse, the stronger the interaction between the two factors. As shown in Figure 8(A) and (B), the 3D response surface plots were steep, and contour lines were elliptical and dense, indicating that the interaction between $(NH_4)_2SO_4$ addition amount and

glucoamylase addition amount, $(NH_4)_2SO_4$ addition amount and inoculation amount had significant effects on the increase rate of ATSI. The results of the response surface analysis were consistent with the results of the analysis of variance.

Verification experiment

The optimal fermentation process predicted by the RSM was as follows: $(NH_4)_2SO_4$ was added in an amount of 0.17%, glucoamylase was added in an amount of 0.87%, and the inoculation amount was 17%. The predicted value under the optimal process was 72.11%. The accuracy of the model prediction was verified under these conditions. The experiment was repeated 3 times, and the average value of the three replicate experiments was 70.34%, which was almost consistent with the predicted value. Therefore, it was feasible and accurate to use this method to optimize the fermentation process for improving the content of ATSI.

Analysis of SI in bean dregs after fermentation

As shown in Table 2, after the bean dregs was fermented by the obtain process, the daidzin completely disappeared, and the contents of glycitein, daidzein, and genistein all increased significantly. It is possible that daidzin was converted to them. However, the increase comtent of ATSI was greater than that of daidzin, indicating that other SI can be converted into ATSI. The content of genistin increased after fermentation, which may be derived from the conversion of other kinds of SI. The experimental results were similar to those of SIRILUN & al. [28] who reported that the contents of glycitein, daidzein, and genistein increased, and the content of genistin changed up and down during the fermentation process.

Analysis of trypsin inhibitors and antigen proteins

The bean dregs were fermented by the obtained process. After determination, the removal rate of trypsin inhibitors reached 81%. As shown in Figure 9, antigenic proteins such as glycinin (11S) and β-conglycinin (7S) were almost completely removed. The obtained fermentation process can remove the main anti-nutritional factors in bean dregs, which greatly improves the safety of fermented SI.

Table 2 Standard curves of SI and analysis of SI before and after fermentation

Species	Standard curves	Before fermentation	After fermentation
Daidzin (mg/kg)	y=34813x+15598 (R^2 = 0.9990)	81.49±2.15	0
Genistin (mg/kg)	y=39374x+325.89 (R^2 = 0.9999)	39.65±2.78	64.47±1.82
Glycitein (mg/kg)	y=48385x-1869.7 (R^2 = 0.9991)	20.11±1.05	46.39±2.33
Daidzein (mg/kg)	y=57564x-1909.2 (R^2 = 0.9993)	33.93±2.21	69.77±2.95
Genistein (mg/kg)	y=41764x-924.95 (R^2 = 0.9989)	76.28±3.54	105.83±4.5
ATSI (mg/kg)		130.32±2.27	221.99±3.26
Increase rate of ATSI (%)			70.34±1.63

Table 3 Design and results of BBD

Number	A	B	C	Increase rate of ATSI (%)	
				Experiment	Predicted
1	0	-1	-1	52.87	54.08
2	-1	1	0	56.66	56.94
3	0	0	0	67.20	67.08
4	1	0	-1	52.38	51.45
5	-1	0	1	66.54	67.47
6	0	-1	1	67.49	67.66
7	0	0	0	68.10	67.08
8	0	1	-1	55.95	55.78
9	0	1	1	66.00	64.79
10	0	0	0	67.11	67.08
11	-1	0	-1	49.42	49.31

12	0	0	0	67.51	67.08
13	0	0	0	65.50	67.08
14	1	-1	0	53.08	52.80
15	-1	-1	0	54.05	52.95
16	1	1	0	46.55	47.65
17	1	0	1	55.78	55.89

Table 4 Analysis of variance of the regression model

Source	Sum of squares	Degree of freedom	Mean square	F value	*p* value (prob >F)
Model	905.30	9	100.59	63.84	< 0.0001
A	44.56	1	44.56	28.28	0.0011
B	0.68	1	0.68	0.43	0.5326
C	225.27	1	255.27	162.01	< 0.0001
AB	20.88	1	20.88	13.25	0.0083
AC	47.06	1	47.06	29.87	0.0009
BC	5.22	1	5.22	3.31	0.1115
A^2	381.86	1	381.86	242.35	< 0.0001
B^2	104.24	1	104.24	66.16	< 0.0001
C^2	9.87	1	9.87	6.26	0.0409
Residual	11.03	7	1.58		
Lack of Fit	7.29	3	2.43	2.60	0.1891
Pure Error	3.74	4	0.93		
Cor Total	916.33	16			

$R^2 = 0.9880$; Asj $R^2 = 0.9725$; Adeq precision = 20.786

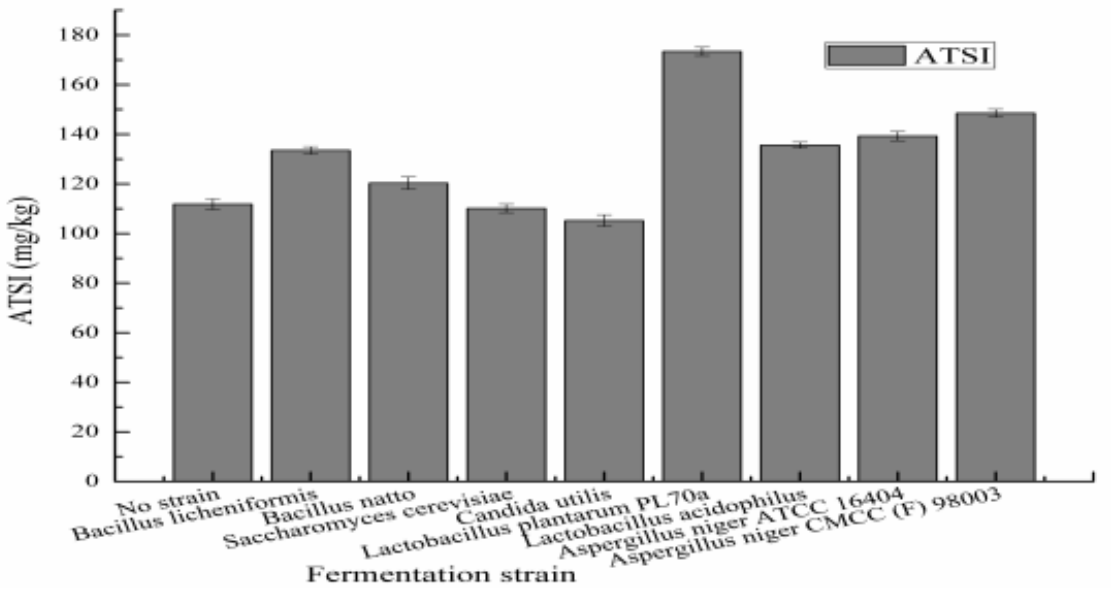

Figure 1. Results of screening dominant strains.

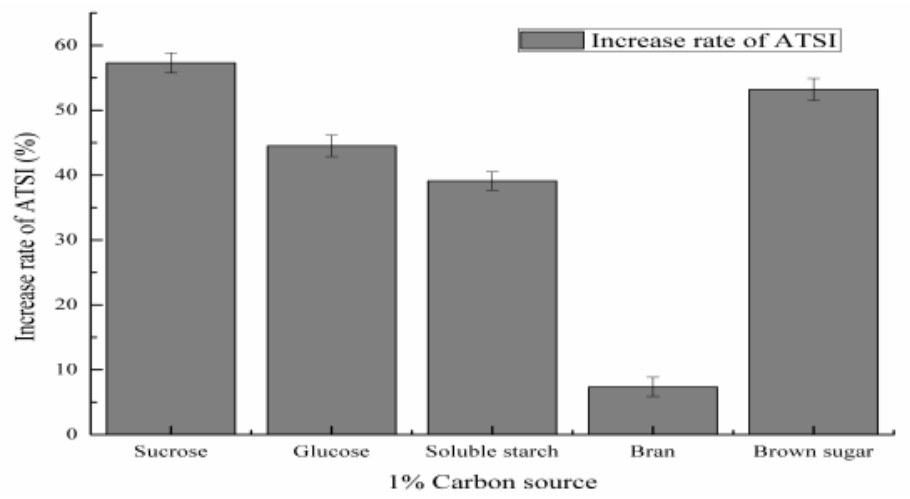

Figure 2. Effect of different carbon sources on the increase rate of ATSI.

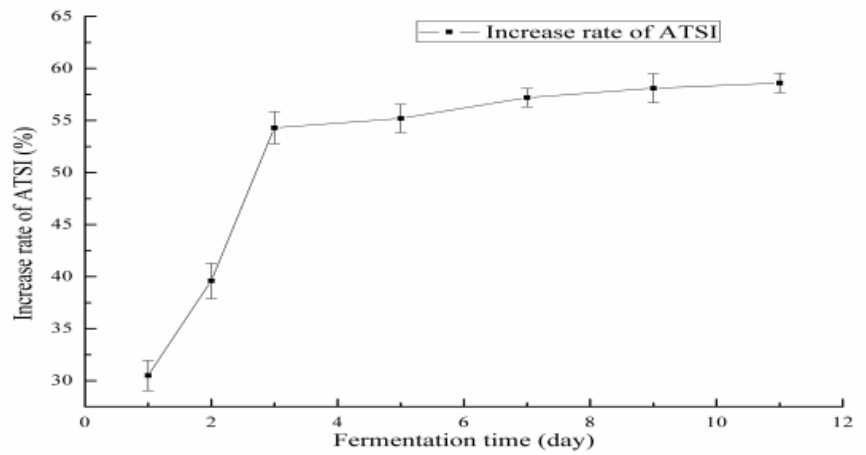

Figure 3. Effect of fermentation time on the increase rate of ATSI.

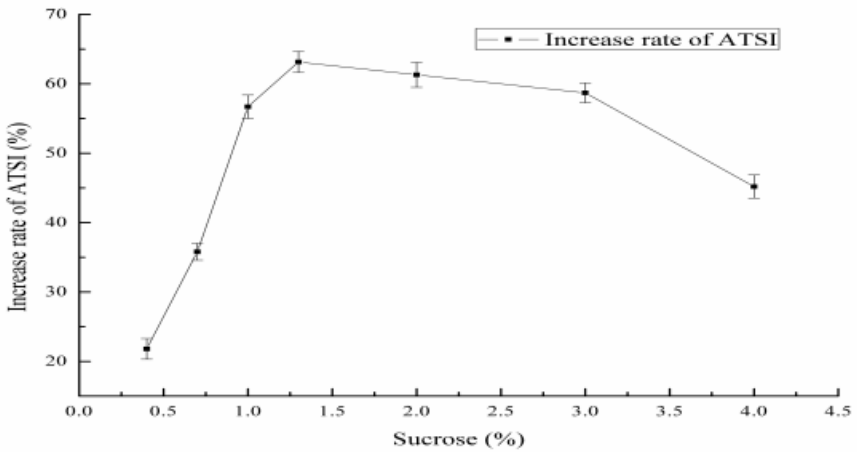

Figure 4. Effect of sucrose addition amount on the increase rate of ATSI.

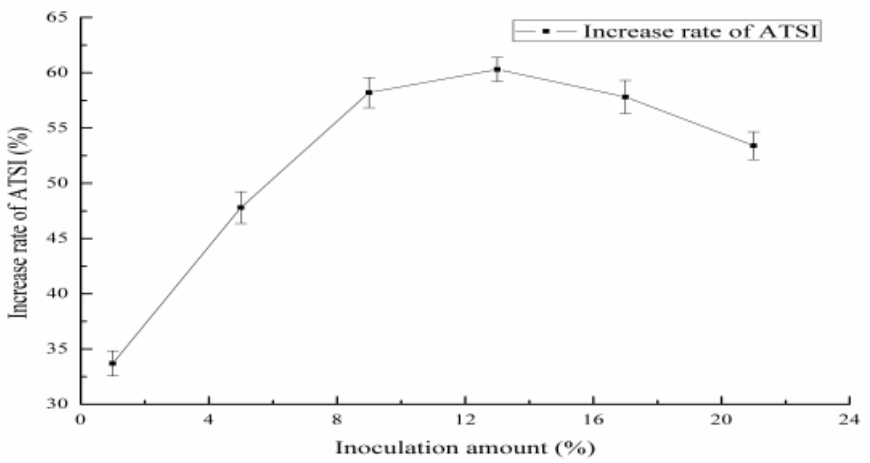

Figure 5. Effect of inoculation amount on the increase rate of ATSI.

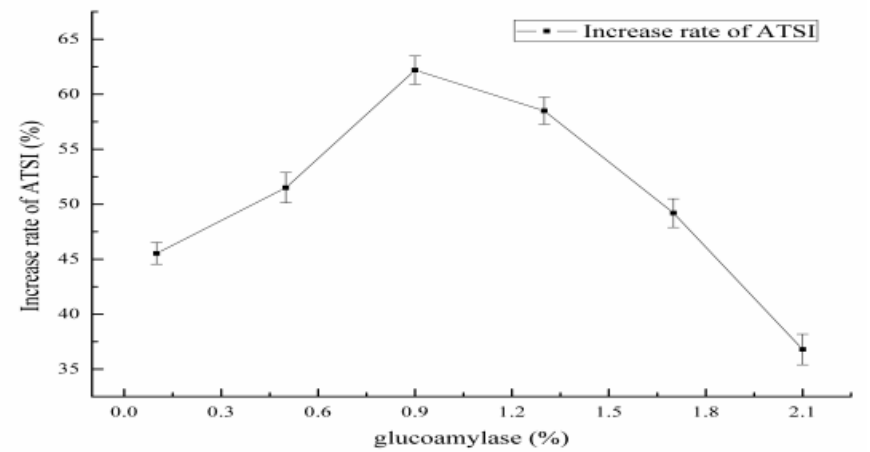

Figure 6. Effect of glucoamylase addition amount on the increase rate of ATSI.

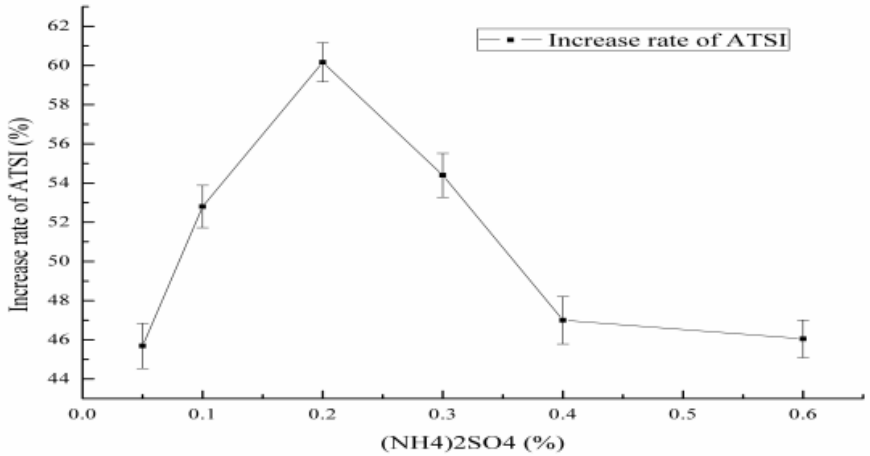

Figure 7. Effect of $(NH_4)_2SO_4$ addition amount on the increase rate of ATSI.

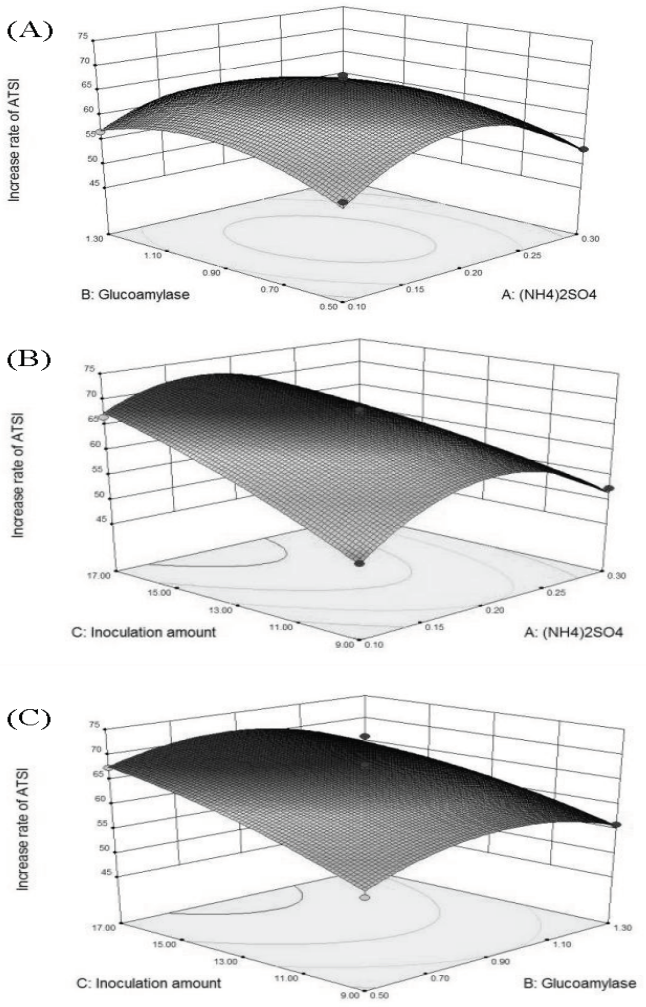

Figure 8. (A): Response surface plots showing the effect of interaction between $(NH_4)_2SO_4$ addition amount and glucoamylase addition amount. (B): Response surface plots showing the effect of interaction between $(NH_4)_2SO_4$ addition amount and inoculation amount. (C): Response surface plots showing the effect of interaction between glucoamylase addition amount and inoculation amount.

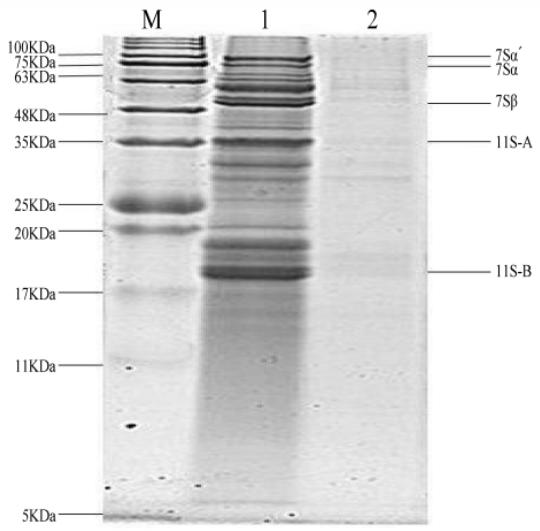

Figure 9. Results of antigen proteins removal. M: Protein marker;
1: unfermented raw material; 2: fermented product.

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Conclusion

The optimal fermentation process was as follows: $(NH_4)_2SO_4$ was added in an amount of 0.17%, glucoamylase was added in an amount of 0.87%, inoculation amount was 17%, sucrose was added in an amount of 1.3%, and fermentation time was 3 days.Under this process, the increase rate of ATSI in bean dregs was 70.34%. At the same time, trypsin inhibitors and antigen proteins in bean dregs were also well removed. The fermented product obtained under the fermentation process can be used as a kind of high-quality SI food. This study also provides a good reference for the processing and application of SI in bean dregs.

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Conflict of interest

The authors declared no conflict of interest. There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

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