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Research article

Improved protease activity of Pixian broad bean paste with cocultivation of *Aspergillus oryzae* QM-6 and *Aspergillus niger* QH-3

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ABSTRACT

Background: The preparation of broad bean koji is a key process in the production of Pixian broad bean paste (PBP). Protease is essential for the degradation of proteins during PBP fermentation. To obtain broad bean koji with high protease activity using the cocultivated strains of *Aspergillus oryzae* QM-6 (*A. oryzae* QM-6) and *Aspergillus niger* QH-3 (*A. niger* QH-3), the optimization of acid and neutral protease activities was carried out using Box–Behnken design with response surface methodology (RSM).

Results: The optimum conditions were found to be as follows: inoculation proportion (X_1), 3:1 (*A. oryzae* QM-6: *A. niger* QH-3, w/w); culture temperature (X_2), 33°C; inoculum size (X_3), 0.5% (w/w); incubation time (X_4), 5 d. The acid and neutral protease activities were 605.2 ± 12.4 U/g and 1582.9 ± 23.7 U/g, respectively, which were in good agreement with the predicted values. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles revealed that the broad bean koji extracellular proteins in the case of cocultivation were richer compared to those in the case of *A. oryzae* QM-6 or *A. niger* QH-3 strain only. In addition, the free amino acids (FAAs) in the fermentation product were 55% higher in the cocultivation process than in that involving only *A. oryzae* QM-6, further confirming the diversity of proteases in the fermentation products.

Conclusions: The optimal conditions of koji-making in PBP were obtained using RSM. The cocultivation of *A. oryzae* and *A. niger* increases the overall enzyme activities in the culture medium and the FAAs content, which would thus have potential application in the PBP industry.

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1. Introduction

Pixian broad bean paste (PBP) is one of the most famous Sichuan condiments regarded as "the Soul of Sichuan Cuisine" because of its palatable taste, reddish-brown luster, and strong savory aroma [1]. PBP is a semi-flowing viscous condiment mostly made from broad beans and is fermented by using koji, which is used as a starter. The three main stages in the production of PBP are the koji-making stage using broad beans, sauce fermentation stage with salt, and post-fermentation stage in the presence of chili. The preparation of broad bean koji is a key process in the production of PBP; the quality of koji is directly related to the extracellular enzyme system of the constituent *Aspergillus* and thus the quality of PBP. In the fermentation stage, the enzymes produced by the growth and reproduction of microorganisms are used to decompose proteins, starch, and other components present in the raw ingredients into smaller molecules, such as polypeptides, free amino acids (FAAs), and sugars [2], which play a vital role in the development of flavor and color of PBP. Hence, improving the enzyme activity of koji is most important for enhancing the quality of PBP.

Aspergillus oryzae (*A. oryzae*) is the key microorganism that has traditionally been used for making koji. It is a filamentous fungus widely used in the fermentation of foods, and can produce amylases and proteases during the koji-making stage. The acid protease activity of *A. oryzae* is very low compared to its neutral protease activity. However, at the initial stage of PBP fermentation, the mash pH gradually decreases to 4.5 as a result of the degradation of proteins and accumulation of organic acids. This decrease in pH inhibits the neutral and alkaline protease activities and thereby suppresses the degradation of proteins [3,4]. Thus, improving the acid protease activity of broad bean koji using a safe and reliable approach is important for the production of rapid-fermented PBP. *Aspergillus niger* (*A. niger*) has been widely used in various biotechnological production processes [5]. The activities of acid proteases produced by *A. niger* are much higher than those produced by *A. oryzae* [6].

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Compared to monocultures, the cocultivation of fungi may result in the generation of enzyme mixtures that could be more efficient for industrial applications [7]. To date, few studies have been conducted on the application of cocultivation for koji-making in the traditional brewing of sauce products. In soy sauce fermentation, the cocultivation of *A. oryzae* HG-26 and *A. niger* HG-35 was reported to result in higher fermentability than the cultivation of *A. oryzae* HG-26 alone, as determined by the comparison of enzyme activities and contents of antioxidants and phenolic compounds [8]. The usage of mixed kojis (*A. oryzae* koji: *A. niger* koji = 3:1) in the production of fish sauce was reported to be effective in accelerating the fermentation process and improved the flavor of fish sauce made with freshwater fish byproducts [6]. Thus, cocultivation of *A. oryzae* and *A. niger* can have a complementary effect on enzyme production. However, to the best of our knowledge, there has been no report on the use of mixed kojis (*A. oryzae* and *A. niger*) in the fermentation of PBP. Thus, cocultivation of *A. oryzae* and *A. niger* maybe a promising approach to producing bean koji with high quality.

Response surface methodology (RSM) is a useful statistical method for determining the optimum design conditions of a multivariable process. It is more beneficial than the "one-variable-at-a-time" method because interactions among the variables are investigated in fewer experiments and in a shorter time [9]. Moreover, RSM provides statistical data on the interaction between variables and gives multiple responses at the same time [10]. It is therefore a powerful statistical technique for the optimization of a complex process [11]. Compared to other models used in RSM, the Box–Behnken designs (BBDs) are efficient and easy experimental designs, and their results are easy to interpret [12].

In the above context, the cocultivation of *A. oryzae* and *A. niger* can effectively improve the acid protease activity while maintaining the level of neutral protease activity of broad bean koji, and would also make up for the shortcomings of low amino acid nitrogen content and a lack of intense umami taste. The objectives of the present study were to investigate the variables (inoculation proportion, culture temperature, inoculum size, and incubation time) determining the protease activity in broad bean koji and to optimize the conditions for improving the protease activity using *A. oryzae* and *A. niger*. Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and FAAs analysis were used to investigate the differences in protein profiles and FAAs contents between the cocultivation cultures and cultures in which only *A. oryzae* or *A. niger* was used.

2. Materials and methods

2.1. Strains, media, and culture conditions

A. oryzae QM-6, with a high yield of neutral protease, and *A. niger* QH-3, with a high yield of acid protease, were screened at the College of Food and Bioengineering, Xihua University, China, from traditional Pixian broad bean koji donated by Sichuan Pixian Douban Co., Ltd. and soy sauce donated by Qianhe Weiye Food Co., Ltd., respectively. The strains were propagated on potato dextrose agar slants at 30°C and stored at 4°C [13]. Spore suspensions of *A. oryzae* QM-6 and *A. niger* QH-3 were diluted to 10^7 spores/mL with 0.9% (w/v) sterile saline solution for inoculation [14]. The seed culture medium was prepared with 8 g of wheat bran, 2 g of soya bean dregs (a mash obtained after the soya bean was crushed), 5 mL of distilled water, and 150 μL of each spore suspension was inoculated in the medium. The cultures were incubated at 30°C for 3 d to get seed koji [15]. Broad bean (*Vicia faba* L.) was purchased from a local market in Chengdu of China. First, the broad beans were put in water at normal temperature for 16 h and were thus peeled. Then the peeled broad beans were boiled for 3 min and cooled in warm water at 40°C for 5 min. The seed koji and wheat flour were mixed evenly, and were then thoroughly mixed with broad beans at a 1:4 (w/w) ratio in triangular flasks and cultured in an incubator for 1~7 d at 24~36°C to get the broad bean koji. The above was the stage of koji-making. For the stage of sauce fermentation, the prepared broad bean koji and 16% saline were mixed at a ratio of 1:1.5, and fermented in an incubator for 30 d at 45°C to obtain the broad bean paste.

2.2. Single-factor experiments

We investigated the effects of inoculation proportion (*A. oryzae* QM-6: *A. niger* QH-3 = 1:1, 2:1, 3:1, 4:1, and 5:1, w/w), culture temperature (24, 27, 30, 33, and 36°C), inoculum size (0.1%, 0.3%, 0.5%, 0.7%, and 0.9%, w/w), and incubation time (1, 2, 3, 4, 5, 6, and 7 d) on the acid and neutral protease activities of broad bean koji. The activities were determined according to the method described by Gao et al. [16] using casein as a substrate. One protease unit (1 U) was defined as being equivalent to 1 μg of tyrosine liberated by 1 mL of enzyme solution at 40°C. The value in each single-factor trial was an average of triplicate estimations.

2.3. BBD and optimization by RSM

On the basis of the results of single-factor experiments, a four-factor, three-level BBD associated with RSM and quadratic programing was employed to determine the optimum conditions of koji-making, and the levels of each factor were obtained according to the results of single-factor experiments.

Keeping the four independent variables, namely inoculation proportion (X_1), culture temperature (X_2), inoculum size (X_3), and incubation time (X_4), at three levels (−1, 0, 1), the activities of acid protease (Y_1) and neutral protease (Y_2) were selected as responses of design experiments. A total of 29 experimental runs were performed to determine the koji-making conditions. The value in each experiment was an average of triplicate estimations. The general form of the mathematical quadratic polynomial equation was as [Equation 1]:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \qquad \text{[Equation 1]}$$

where Y is the response variable (protease activity); X_i and X_j are the independent variables; β_0 is the coefficient of the model intercept; β_i is the linear regression coefficient; β_{ii} is the quadratic term coefficient, and β_{ij} is the interaction coefficient for the second-order terms.

The experimental design data were analyzed using Design Expert 8.0.6 software, the quadratic polynomial model was obtained by multiple regressions, and the significant terms in the model were evaluated by the analysis of variance (ANOVA).

2.4. SDS-PAGE analysis of the protein profiles

The koji were obtained under the optimal koji conditions using the cocultivation of *A. oryzae* QM-6 and *A. niger* QH-3 or the cultivation of only *A. oryzae* QM-6 or *A. niger* QH-3. The amounts of *A. oryzae* or *A. niger* separately used in the cultivation were the same as the total amounts of *A. oryzae* and *A. niger* used in cocultivation. The koji were extracted using 0.1-M sterile saline solution (koji:solvent = 10 g:40 mL) at 40°C for 1 h. The extract was first centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was further centrifuged at 12,000 rpm for 25 min at 4°C. Finally, the spores in the supernatant were removed by a 0.45-μm microporous membrane, and the filtrate was collected as crude protein solution [17]. A total of 50 μL of the solution was used for SDS-PAGE. Extracellular proteins were resolved on 12% polyacrylamide gels, and the protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 (CBB R-250) after electrophoresis [18].

2.5. Amino acid analysis

The acid-hydrolysis method was used to determine the FAAs content. The samples obtained after sauce fermentation were mixed evenly with 10-mL 6-mol/L hydrochloric acid and vacuumed, then hydrolyzed at 110°C for 22 h and, finally, cooled. The collection was fixed to 50 mL, and 1 mL of the filtrate was pipetted into a 5-mL volumetric flask after filtration. The filtrate was dried at 50°C, and the residue was hydrolyzed with 1-mL hydrochloric acid, dried and then evaporated. The collection was dissolved in 1-mL citric acid buffer and filtered through a pinhole filtration membrane (0.22 μm), subsequently, applied to an automatic amino acid analyzer (L-8900, Hitachi, Japan). Peak quantification was accomplished by determining peak areas from the instrument software in comparison to FAAs standards [19,20].

2.6. Statistical analysis

All the results are presented as means ± standard deviation (SD). Statistical analyses were carried out using Origin 8.0 software.

3. Results and discussion

3.1. Single-factor experimental analysis

Experiments were designed to evaluate the effects of four factors (inoculation proportion, culture temperature, inoculum size, and incubation time) on the activities of acid and neutral proteases. The effects of these parameters on the protease activity of broad bean koji are illustrated in Fig. 1. The evaluation of the effects of inoculation proportion (*A. oryzae* QM-6:*A. niger* QH-3 varied from 1:1 to 5:1, w/w) on the protease activities revealed that the activities of both the proteases increased as the inoculation proportion increased from 1:1 to 3:1 and then decreased with a further increase from 3:1 to 5:1.

Temperature is one of the important factors affecting the growth and reproduction of microorganisms. Furthermore, the culture temperature affects the activity and stability of proteases. It is closely related to the germination of *Aspergillus* spores, growth of hyphae, respiratory metabolism of *Aspergillus*, enzyme activity of koji, and proliferation of microorganisms during the process of koji-making. Therefore, temperature is one of the most important factors in the koji technique [21]. The effects of culture temperature on the activities of acid and neutral proteases were investigated over a temperature ranging from 24 to 36°C. The protease activity increased with the increase in culture temperature from 24 to 33°C, whereas it significantly decreased after a further increase in temperature.

The inoculum size directly affects the enzyme activity and koji-making. The effects of an increase in inoculum size from 0.1% to 0.9% on the acid and neutral protease activities were investigated. The protease activity increased with the increase in inoculum size from 0.1% to 0.5% and then decreased with a further increase in inoculum size from 0.5% to 0.9%, possibly because of the exhaustion of nutrients and production of a large amount of metabolic waste, which deteriorated the environment in which the microorganisms grew. Moreover, because of limited nutrients that were insufficient to sustain the growth of the strains, large numbers of dormant spores were formed, which ultimately reduced the enzyme activity [22]. These results were similar to those reported by Bansal et al. [23].

The incubation time has an important influence on the growth and reproduction of microorganisms [24]. Effects on the protease activity were investigated by varying the incubation time from 1 to 7 d. The activities of both the proteases were observed to increase with the increase in incubation time from 1 to 5 d; the acid protease activity was significantly decreased when the incubation time was further

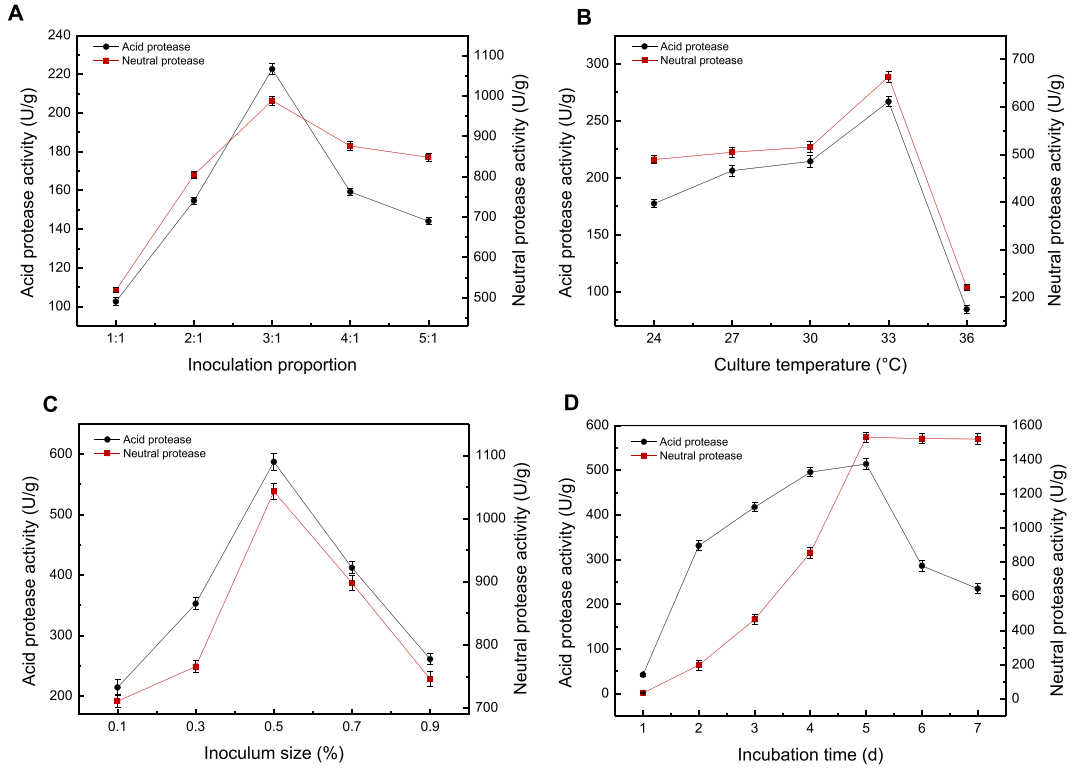

Fig. 1. Effects of culture conditions on the protease activity of broad bean koji. (A) Effects of inoculation proportion in the range of 1:1–5:1(w/w) at a fixed incubation time of 5 d, culture temperature of 30°C, and inoculum size of 0.5%; (B) effects of culture temperature in the range of 24–36°C at a fixed incubation time of 5 d, inoculum size of 0.5%, and inoculation proportion of 3:1; (C) effects of inoculum size in the range of 0.1–0.9% at a fixed incubation time of 5 d, culture temperature of 30°C, and inoculation proportion of 3:1 (w/w); and (D) effects of incubation time in the range of 1–7 d at a fixed culture temperature of 30°C, inoculum size of 0.5%, and inoculation proportion of 3:1.

increased from 5 to 7 d, whereas the activity of neutral protease remained stable.

Based on the above results, different inoculation proportions (2:1, 3:1, 4:1), culture temperatures (30, 33, 36°C), inoculum sizes (0.3, 0.5, 0.7%), and incubation times (4, 5, 6 d) were selected for further optimizing the activities of acid and neutral proteases using RSM.

3.2. Model fitting

BBD evaluates the nonlinear relationship between test indicators and influencing factors. It has received much attention in the food industry in recent years because it requires fewer factors and experiments [25]. In this study, a BBD was applied with four independent parameters at three levels. The factors and levels of response surface experiments are shown in Table 1. Based on the regression analysis of the experimental data, the polynomial models obtained were as [Equation 2] and [Equation 3]:

$$Y_1 = 590.95 - 12.25X_1 - 10.57X_2 + 61.59X_3 - 10.03X_4 - 1.89X_1X_2 - 53.71X_1X_3 - 8.99X_1X_4 + 11.26X_2X_3 + 5.99X_2X_4 - 33.79X_3X_4 - 199.84X_1^2 - 279.44X_2^2 - 195.34X_3^2 - 295.66X_4^2 \quad \text{[Equation 2]}$$

$$Y_2 = 1484.93 + 28.16X_1 - 96.96X_2 - 63.53X_3 - 103.25X_4 + 94.93X_1X_2 - 46.47X_1X_3 - 107.50X_1X_4 - 121.90X_2X_3 + 80.36X_2X_4 - 61.63X_3X_4 - 566.34X_1^2 - 281.74X_2^2 - 488.93X_3^2 - 299.87X_4^2 \quad \text{[Equation 3]}$$

ANOVA was performed to evaluate the regression model and relationship between the response and the variables. The results of ANOVA for acid and neutral protease activities are shown in Table 2 and Table 3, respectively. Values of "Prob > F" < 0.0001 indicated that the models were statistically significant. The p-values of the lack of fit greater than 0.05 indicated a good fit to the simulation of the quadratic regression model. Moreover, the coefficient of determination (R^2) showed a good fit between the dependent variable and the independent variable, and the adjusted R^2 and predicted R^2 were close to 1, indicating that the observed experimental data had a high degree of correlation with the predicted values [26,27]. Furthermore, the coefficient of variation (CV) values was less than 15%, indicating a high degree of precision and high reliability of the experimental data [28].

3.3. Analysis of three-dimensional (3D) response surfaces

RSM is a widely used experimental optimization method [29]. RSM can use the quadratic regression equation to fit the functional relationship between multiple factors and multiple response values, and seeks the optimal process parameters through the regression equation mathematical model [30,31]. The interactive effect of any two of the four independent variables on the dependent variable was illustrated graphically by using a 3D response surface. The significant interactive effects of inoculation proportion, culture temperature, inoculum size, and incubation time on the acid and neutral protease activities are shown in Fig. 2 and Fig. 3, respectively. As shown in Fig. 2, the acid protease activity first increased with the increase in inoculation proportion, culture temperature, inoculum size, and incubation time, but decreased dramatically beyond the optimum value. The effects of inoculation proportion and inoculum size on the neutral protease activity first showed an increasing trend, which was followed by a decrease (Fig. 3); however, the neutral protease activity increased slowly with the increase in culture temperature and incubation time.

Table 1
Factors and levels of response surface experiments.

Variables	Coded	Range and levels		
		Low (−1)	Middle (0)	High (+1)
Inoculation proportion	X_1	2:1	3:1	4:1
Culture temperature (°C)	X_2	30	33	36
Inoculum size (%)	X_3	0.3	0.5	0.7
Incubation time (d)	X_4	4	5	6

Table 2
Analysis of variance (ANOVA) of the quadratic model for acid protease activity.

Source	Sum of squares	df	Mean square	F value	p-Value Prob > F	Significance
Model	1.107E+006	14	79,056.77	1021.79	<0.0001	***
X_1	1801.49	1	1801.49	23.28	0.0003	***
X_2	1385.55	1	1385.55	17.91	0.0008	***
X_3	45,516.95	1	45,516.95	588.29	<0.0001	***
X_4	1207.86	1	1207.86	15.61	0.0014	**
X_1X_2	14.25	1	14.25	0.18	0.6743	
X_1X_3	11,540.35	1	11,540.35	149.16	<0.0001	***
X_1X_4	323.58	1	323.58	4.18	0.0601	
X_2X_3	507.33	1	507.33	6.56	0.0226	*
X_2X_4	139.15	1	139.15	1.80	0.2013	
X_3X_4	4568.30	1	4568.30	59.04	<0.0001	***
X_1^2	2.590E+005	1	2.590E+005	3348.05	<0.0001	***
X_2^2	5.065E+005	1	5.065E+005	6546.36	<0.0001	***
X_3^2	2.475E+005	1	2.475E+005	3199.01	<0.0001	***
X_4^2	5.670E+005	1	5.670E+005	7328.74	<0.0001	***
Residual	1083.20	14	77.37			
Lack of Fit	988.64	10	98.66	4.18	0.0902	
Pure Error	94.56	4	23.64			
R^2	0.9990					
Adj R^2	0.9980					
Pre R^2	0.9947					

X_1 = Inoculation proportion, X_2 = Culture temperature (°C), X_3 = Inoculum size (%), X_4 = Incubation time (d); Level of significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; and coefficient of variation (CV) = 4.64%.

3.4. Verification of the model

The optimum conditions of koji-making determined from the model were as follows: inoculation proportion (X_1), 3.00; culture temperature (X_2), 32.79°C; inoculum size (X_3), 0.51%; and incubation time (X_4),

Table 3
Analysis of variance (ANOVA) of the quadratic model for neutral protease activity.

Source	Sum of squares	df	Mean square	F value	p-Value Prob > F	Significance
Model	3.475E+006	14	2.482E+005	65.34	<0.0001	***
X_1	9513.35	1	9513.35	2.50	0.1359	
X_2	1.128E+005	1	1.128E+005	29.70	<0.0001	***
X_3	48,428.92	1	48,428.92	12.75	0.0031	**
X_4	1.279E+005	1	1.279E+005	33.68	<0.0001	***
X_1X_2	36,050.43	1	36,050.43	9.49	0.0081	**
X_1X_3	8636.73	1	8636.73	2.27	0.1538	
X_1X_4	46,221.35	1	46,221.35	12.17	0.0036	**
X_2X_3	59,442.10	1	59,442.10	15.65	0.0014	**
X_2X_4	25,830.60	1	25,830.60	6.80	0.0207	*
X_3X_4	15,194.88	1	15,194.88	4.00	0.0653	
X_1^2	2.080E+006	1	2.080E+006	547.68	<0.0001	***
X_2^2	5.149E+005	1	5.149E+005	135.55	<0.0001	***
X_3^2	1.307E+006	1	1.307E+006	344.14	<0.0001	***
X_4^2	3.427E+005	1	3.427E+005	90.23	<0.0001	***
Residual	53,181.22	14	3798.66			
Lack of Fit	49,448.73	10	4944.87	5.30	0.0611	
Pure Error	3732.49	4	933.12			
R^2	0.9849					
Adj R^2	0.9699					
Pre R^2	0.9176					

X_1 = Inoculation proportion, X_2 = Culture temperature (°C), X_3 = Inoculum size (%), X_4 = Incubation time (d); Level of significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; and coefficient of variation (CV) = 7.22%.

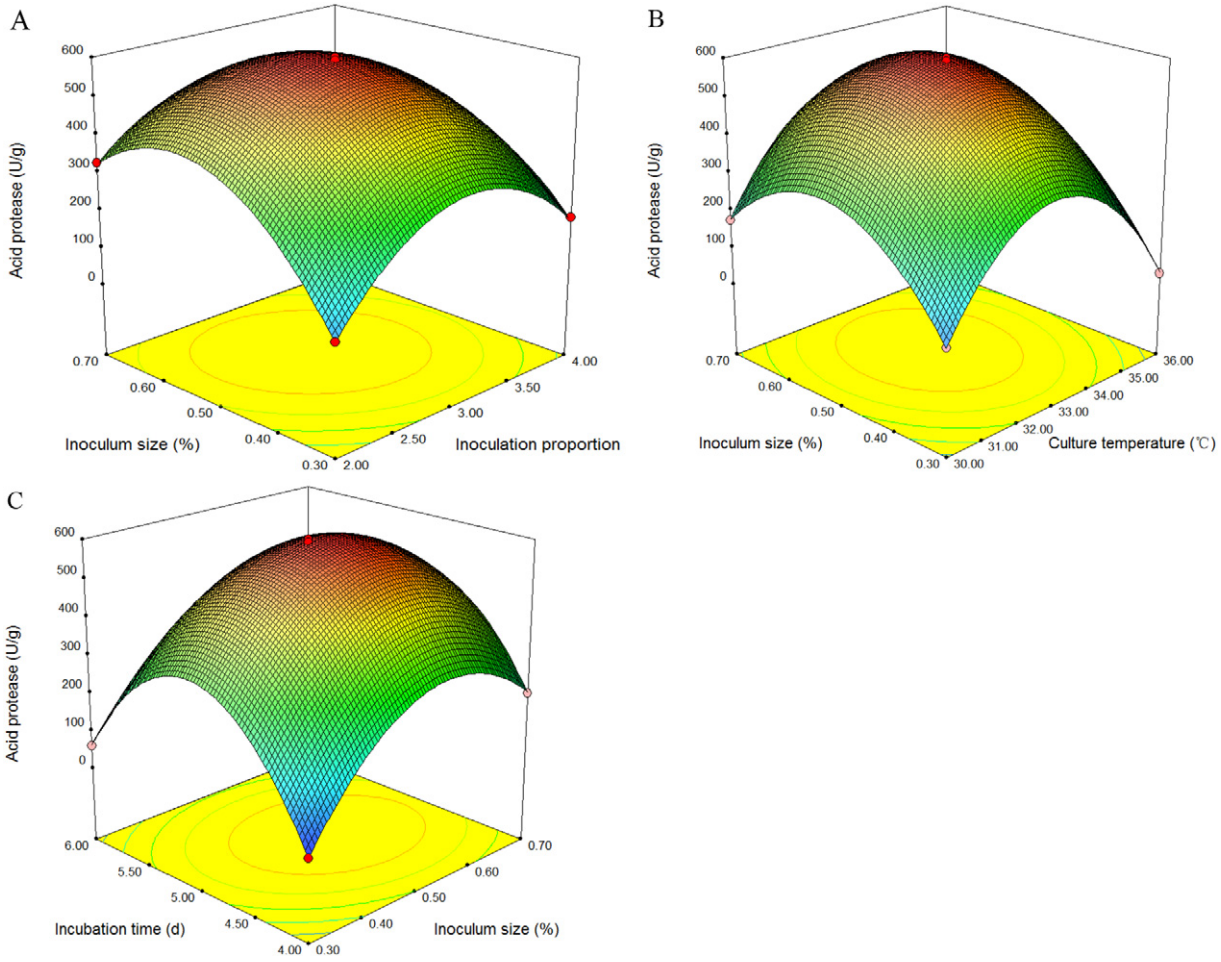

Fig. 2. Response surface diagrams showing the effects of the mutual interactions between two independent variables on the acid protease activity from broad bean koji. (A) Effects of inoculum size and inoculation proportion; (B) effects of inoculum size and culture temperature; and (C) effects of incubation time and inoculum size.

4.93 d. For practical applicability, the conditions were modified as follows: inoculation proportion (X_1), 3; culture temperature (X_2), 33°C; inoculum size (X_3), 0.5%; and incubation time (X_4), 5 d. Under optimal conditions, the acid and neutral protease activities were 605.2 ± 12.4 U/g and 1582.9 ± 23.7 U/g, which were fairly close to the predicted values of 592.6 U/g and 1492.4 U/g, respectively. These results demonstrate that the established polynomial model was statistically reliable.

3.5. Effect of cocultivation on the profiles of whole-cell extracellular proteins

Some articles have reported on the positive effect of cocultivations of fungi on the production of hydrolytic enzymes [32,33,34]. The SDS-PAGE profiles of extracellular proteins under the optimum koji-making conditions when using *A. oryzae* QM-6 and *A. niger* QH-3 alone or in cocultivation are presented in Fig. 4. The results showed considerable differences in patterns of proteins in three conditions. The molecular weights of the extracellular proteins in the product of broad bean koji prepared using the *A. oryzae* QM-6 strain ranged from 20.0 to 45.0 kDa (Fig. 4A), and the number of protein bands was gradually increased from 1 to 7 d. The molecular weights of the extracellular proteins using the *A. niger* QH-3 strain ranged from 45.0 to 66.2 kDa (Fig. 4B), and there was no significant change in the extracellular total protein band from 1 to 7 d. In contrast, when *A. oryzae* QM-6 and *A. niger* QH-3 were cocultivated, the molecular weights of the extracellular proteins ranged from 20.0 to 62.0 kDa (Fig. 4C), and no significant change was observed in the number of protein bands when the incubation time was increased from 1 to 7 d; however, the number of extracellular proteins in the case of cocultivation was higher than in the case where only *A. oryzae* QM-6 or *A. niger* QH-3 was used, indicating that the process of koji-making using the cocultivation of the two increased the abundance of high molecular weight proteins. This finding is consistent with Hu et al. [7]. The positive results demonstrated that the cocultivation of *A. oryzae* QM-6 and *A. niger* QH-3 contributed to the overall enzyme production in koji, which is dominant to the brewing of PBP. Yoshino-Yasuda et al. [35] has reported that more secreted enzymes of *A. oryzae* are regarded as basal factors affecting the quality of fermented soybean paste. However, the ability of cocultivation with *A. oryzae* QM-6 and *A. niger* QH-3 to improve enzyme production has not been proven in this study and that will need a further investigation.

3.6. Comparison of FAA production

FAA is one of the main factors that is closely related to the flavor of PBP. The degradation of proteins triggers the formation of FAAs, so the formation and changes in the content of FAAs can reflect the degradation process of protein [36]. The amounts of FAAs produced in the fermentation using cocultivation of *A. oryzae* QM-6 and *A. niger* QH-3 was 55% higher than that produced when only *A. oryzae* QM-6 was used (Table 4). The enriched protease system in the cocultivation condition was more conducive to protein degradation and FAAs production. The eight essential amino acids were detected, except for tryptophan, and the concentrations of the remaining seven essential

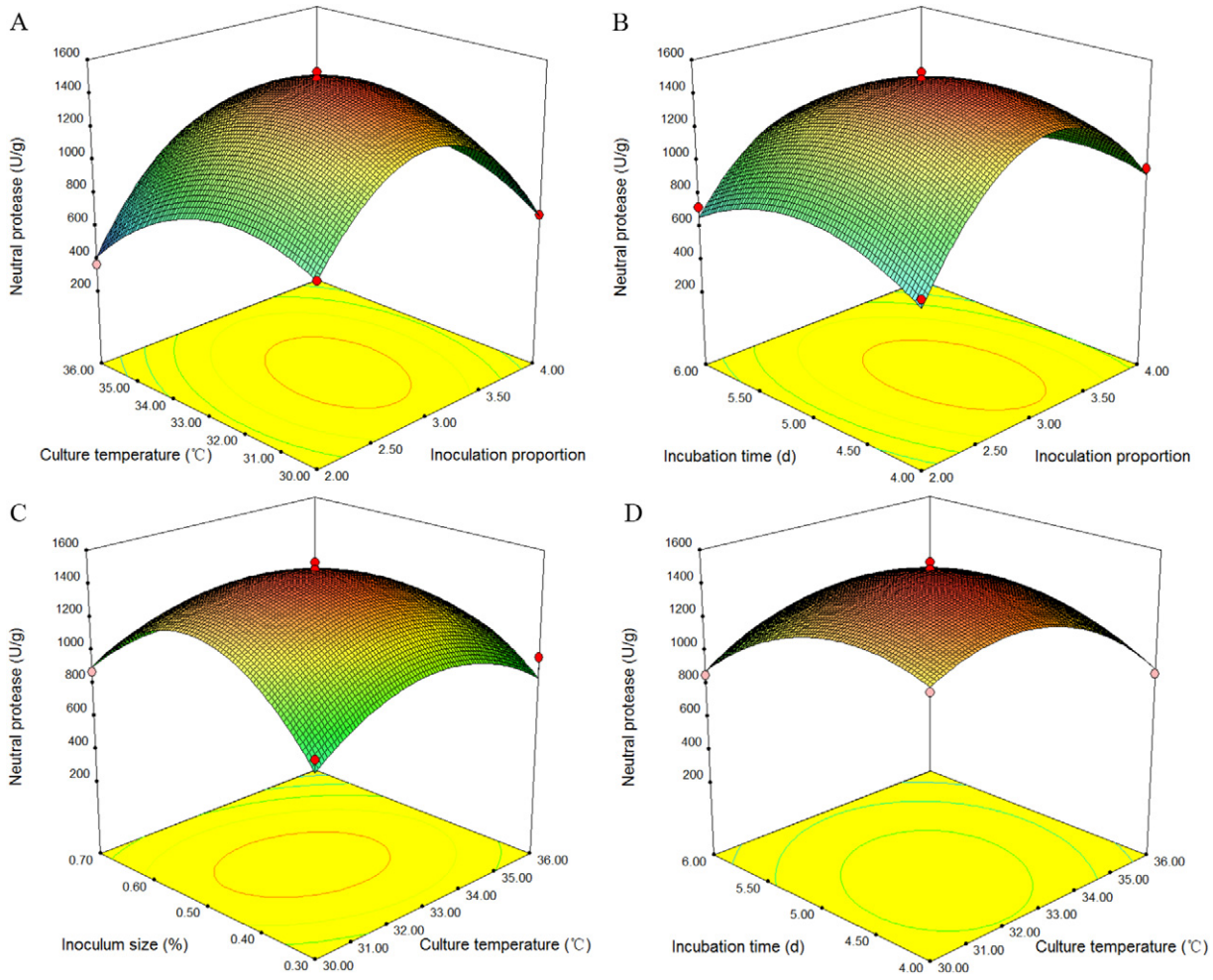

Fig. 3. Response surface diagrams showing the effects of the mutual interactions between two independent variables on the neutral protease activity from broad bean koji. (A) Effects of culture temperature and inoculation proportion; (B) effects of incubation time and inoculation proportion; (C) effects of inoculum size and culture temperature; and (D) effects of culture temperature and incubation time.

amino acids, namely threonine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine, in the fermentation with *A. oryzae* QM-6 alone or in combination with *A. niger* QH-3 were 1.506 and 2.319 g/100 g, respectively. This indicated that the products obtained by fermentation using cocultivation have higher nutritional value. Typically, the initial fermentation process of PBP is carried out under a low pH. Thus, acidic proteases are especially important for the hydrolysis of proteins. Alkaline and neutral proteases are the predominant proteases produced by *A. oryzae* [15]. It is probably due to the complementation of these proteases produced during the cocultivation of *A. oryzae* and *A. niger* that makes the protein degradation more complete, and thus, the amino acid content is richer. In addition, among the FAAs, the content of glutamic acid in the fermentation product obtained by cocultivation was significantly increased compared to that of the product obtained after fermentation with *A. oryzae* QM-6 alone. A similar result was reported for the cocultivation of *A. oryzae* koji and *A. niger* koji with respect to the higher glutaminase activity, which may be responsible for the higher content of glutamic acid [8]. Glutamic acid has been reported to improve the overall flavor of soybean paste [37].

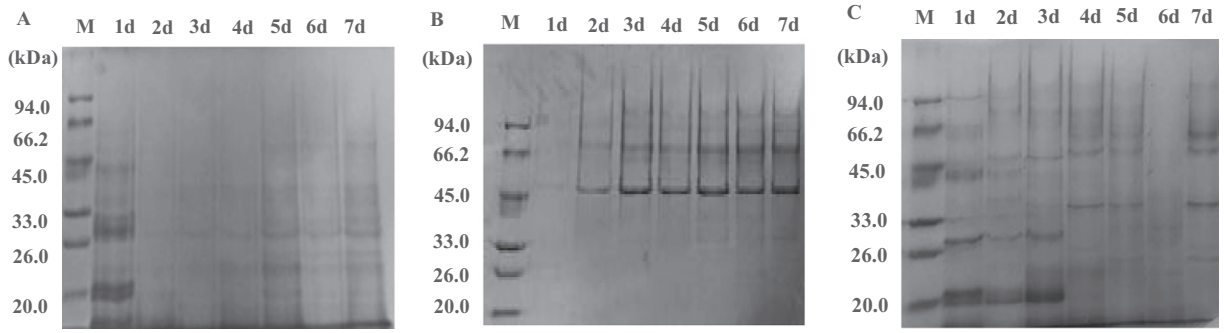

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of extracellular proteins obtained in the two methods of koji-making. (A) When only *A. oryzae* QM-6 strain was used; (B) when only *A. niger* QH-3 strain was used; and (C) when both *A. oryzae* QM-6 and *A. niger* QH-3 were used (*A. oryzae* QM-6: *A. niger* QH-3 = 3:1, w/w). M = Molecular weight markers.

Table 4
Contents of free amino acids (FAAs) after sauce fermentation with *A. oryzae* QM-6 or coculture with *A. oryzae* QM-6 and *A. niger* QH-3.

FAA (g/100 g)	Ao	Ao + An
Asp	0.430 ± 0.030	0.760 ± 0.026*
Thr	0.170 ± 0.002	0.260 ± 0.017*
Ser	0.210 ± 0.007	0.310 ± 0.015*
Glu	0.710 ± 0.029	1.120 ± 0.071*
Gly	0.230 ± 0.022	0.330 ± 0.011*
Ala	0.280 ± 0.027	0.360 ± 0.017*
Cys	0.028 ± 0.003	0.021 ± 0.002
Val	0.250 ± 0.017	0.380 ± 0.017*
Met	0.036 ± 0.002	0.059 ± 0.005*
Ile	0.210 ± 0.014	0.330 ± 0.012*
Leu	0.380 ± 0.013	0.550 ± 0.028*
Tyr	0.073 ± 0.005	0.220 ± 0.019*
Phe	0.200 ± 0.008	0.340 ± 0.009*
Lys	0.260 ± 0.021	0.400 ± 0.015*
His	0.100 ± 0.014	0.160 ± 0.010
Arg	0.091 ± 0.007	0.087 ± 0.006
Pro	0.160 ± 0.010	0.230 ± 0.013*
Total	3.818 ± 0.120	5.917 ± 0.225*

The values are presented as mean ± standard deviation (n = 3). *Indicates significant differences in the same row ($p < 0.05$). Ao = *Aspergillus oryzae* QM-6, An = *Aspergillus niger* QH-3.

4. Conclusion

In this study, the activities of acid and neutral proteases on the cocultivation of *A. oryzae* QM-6 and *A. niger* QH-3 during the production of PBP were optimized using RSM. Based on the results of single-factor experiments, the BBD was used to optimize the koji-making conditions, with respect to inoculation proportion, culture temperature, inoculum size, and incubation time. Moreover, the extracellular enzyme in the case of cocultivation were richer compared to those present when *A. oryzae* QM-6 or *A. niger* QH-3 was used alone, as a consequence of which the FAAs content, particularly the glutamate content, was higher in the case of cocultivation. To the best of our knowledge, this is the only study where the application of cocultivation of *A. oryzae* and *A. niger* for the production of PBP has been demonstrated. Our work presents a new method of producing high-protease koji, which will help enhance the economic efficiency of PBP production.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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