Optimization of peptidase production conditions in koji making

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The effects of the parameters involved in koji making were investigated with a view to increase amino peptidase activity. In order to increase amino peptidase during the koji making, the optimum proportion of wheat bran (35-45%)(w/w), the addition water ration (90-110%)(v/w), the raw material cooking time (15-25 min) and for koji making (38-46 h) was obtained by using response surface. The central composite design (CCD) used for the analysis of treatment combinations showed that the experimental results were in agreement with the second-order polynomial regression model well with R² = 0.99 (p<0.05). Based on surface and contour plots, optimum conditions for amino peptidase activity in koji making were wheat bran proportion 41% (w/w), water ratio100% (v/w), raw material cooking for 21 min and koji making for 44 h.

Key words: Koji making, amino peptidase, response surface methodology, optimization.

INTRODUCTION

Koji making is the most important step in the processing of soy sauce fermentation. Its purpose is to produce enzymes. The efficient production of enzymes concerned, especially the peptidases, is very important in the economy of soy sauce manufacturing. Peptidases from Aspergillus oryzae were found to be important in the digestion of the peptides to yield free amino acids. Especially, the contribution rate of leucine amino peptidase II in Glu liberation from soybean proteins was about 79% (Nakadai et al., 1972). However, it was found that many researchers focused on the character of enzymes such as proteinase, amylase, cellulose, xylanase, glutaminase and lipase in soy sauce koji (Yuichi et al., 2002; Tadaaki et al., 1999), and that many researchers focused on the effect of various material on proteinase production in soy sauce koji (Ling et al., 1996), but little researches were found on peptidase production in wheat bran koji (Tadanobu et al., 1988). And some studies were found on using optimal method to improve enzymes production by A. oryzae in solid-state fermentation (Hamidi-Esfahani et al., 2004; Georgi et al., 2007), but the studies are rarely found on using optimal method on improving amino peptidase production in koji making process.

Response surface methodology (RSM) is a statistical and mathematical technique which is useful in developing, improving, and optimizing processes. In which a response influenced by several variables and the objective is to optimize this response (Myers et al., 2002). It is a designed regression analysis meant to predict the value of a dependent variable based on the controlled values of the independent variables (Schutz et al., 1983). In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model which describes the chemical or biochemical processes (Thanongsak et al., 2011; Myers et al., 2002; Anjum et al., 1997). RSM has been very popular for optimization studies in recent years. There are so many works based on the application of RSM in chemical and biochemical process (Hsiao et al.,2011; Júlio et al.,2005; Febe et al., 2003).

Although lots of studies concerning koji making and enzyme production have been reported in the literatures, there is little information on optimizing the koji making process.
Therefore, this paper deals with koji making condition optimize the following parameters: wheat bran proportion, water ratio, raw material cooking time and koji making time. The aim of this study is to optimize the koji making for amino peptidase production in solid-state fermentation of the strain *A. oryzae*, by using the methods of mathematical modeling and statistical processing of the results.

**MATERIALS AND METHODS**

**Microorganism**

The experiments were carried out with the strain *A. oryzae* AS3.951 (China Center of Industrial culture collection, Beijing, China) collected from the company Wuxi Flavoring Food Co. Ltd (Wuxi, Jiangsu, China). The preservation and sporulation of the strain were done on a solid nutrient medium (Difco, Shanghai, China) with the following compositions (g/L): potato starch 4.0, dextrose 20.0, and agar 15.0. The medium was sterilized at 12°C for 20 min. The inoculated test-tubes with slant agar were incubated at 30°C for 7 days, and stored at 4°C for 3–4 months.

**Raw materials and chemicals**

Wheat bran and soy bean were purchased from Sanliqiao Market (Wuxi, Jiangsu, China). Casein and soluble starch were purchased from Merck (Shanghai, China). Tyrosine and L-leucine-p-nitroanilide were purchased from Sigma (Shanghai, China).

**Process for koji making**

To prepare seed koji, wheat bran and defatted soybean were mixed with 80% water and then cooked at 121°C for 30 min. The cooked wheat bran and defatted soybean mixture was inoculated with spores of *A. oryzae* and incubated at 30°C for 3 days, then dried with hot air at 40°C and ground into powders. Essentially, the procedures employed in the soy sauce factories in China were only followed to prepare the soy sauce koji. After moisture adjusted and 121°C cooked, raw materials were mixed with seed koji in a ratio of 1:1500 (w/w) and then placed on koji trays (35×23.5×7 cm) with a thickness of 2 cm. They were kept in a koji preparation room for hours. The relative humidity and temperature were maintained at >90% and 30°C respectively. After 24 h of incubation, the koji were stirred. The temperature was lowered to 25°C in the rest of incubation time.

**Enzyme extraction**

The crude enzyme from the fermented material was extracted by simple contact method. The fermented substrate (5 g) was mixed thoroughly with distilled water (100 ml) by shaking for 1 h at 40°C. At the end of the extraction, the suspension was centrifuged at 3000 rpm for 10 min and the supernatant was collected and used as the crude enzyme for further research.

**Amino peptidase assay**

Amino peptidase activity was assayed according to the procedure described by former research (Tan, 1990) with some modifications.

The standard reaction mixture contained 50 mM glycine–NaOH (pH 8.5), 1 mM L-leucine-p-nitroanilide, and an appropriate amount of the enzyme solution. After 10 min incubation at 50°C, the reaction was stopped by the addition of acetic acid to a final concentration of 30% and the resultant color was measured at 504 nm. The volume of this system at the end of reaction is 10 mL. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1µmol of L-leucine-p-nitroanilide per minute with p-nitroanilide under the assay condition.

**Protease and α-amylase assay**

Protease activity was quantified by the production of tyrosine after hydrolyzing 20 g/L casein in 0.2 M pH 7 phosphate buffers for 10 min. The amount of protease for the production of one milligram tyrosine in one minute at 40°C was defined as one unit of activity (U) under the assay conditions.

Alpha-Amylase activity was determined as described by Okolo (Okolo, 1995). The reaction mixture consisted of 1.25 ml 1% (w/v) soluble starch solution, 0.25 ml 0.1 M sodium acetate buffer (pH 5.0), 0.25 ml distilled water, and 0.25 ml properly diluted crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid method (Miller, 1959). Appropriate blanks were used. One unit (U) of α-amylase is defined as the amount of enzyme releasing 1µmol glucose equivalent per minute under the assay conditions.

**Statistical analysis**

To determine the influence of parameters in koji making, a central composite design (CCD), with k = 4, was used in order to generate 31 treatment combinations (including seven replicates at the centre point with each value coded as 0), with wheat bran proportion (*X_1*), water ration(*X_2*), raw material cooking time (*X_3*), koji making time (*X_4*) as independent variables. Five levels of each variable were chosen, the upper and lower limits of them, set to be in the range according to the former experimental results and production parameters. SAS for Windows V8 software (Cary, NC, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by t-test; the second-order model equation was determined by Fisher’s test. The variance explained by the model is given by the multiple coefficient of determination, R2.

**RESULTS AND DISCUSSION**

The experimental design of the coded (*x*) and actual (*X*) levels of variables and the coded and actual values of the four independent variables together with the responses are shown in Table 1. The test factors were coded according to the following regression equation:

\[ x_i = \frac{X_i - X_0}{\Delta X_i} \]  

(1)

Where *x_i* is the coded value and *X_i* is the actual value of the *i*th independent variable, *X_0* is the actual value at the center point, and \( \Delta X_i \) is the step change value. The responses functions (*y*) were amino peptidase activity. These values were related to the coded variables \( x_i, i = 1 \)
to 4) by a second degree polynomial using equation:

\[ y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_11x_1^2 + b_12x_1x_2 + b_22x_2^2 + b_33x_3^2 + b_44x_4^2 + b_13x_1x_3 + b_14x_1x_4 + b_23x_2x_3 + b_24x_2x_4 + b_34x_3x_4 
+ b_12x_1x_2 + b_13x_1x_3 + b_14x_1x_4 + b_22x_2^2 + b_23x_2x_3 + b_24x_2x_4 + b_33x_3^2 + b_34x_3x_4 + b_44x_4^2 \]

The coefficients of the polynomial were represented by \( b_0 \) (constant term), \( b_1, b_2, b_3 \) and \( b_4 \) (linear effects), \( b_{11}, b_{22}, b_{33} \) and \( b_{44} \) (quadratic effects), and \( b_{12}, b_{13}, b_{14}, b_{23}, b_{24} \) and \( b_{34} \) (interaction effects). The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significance of all terms in the polynomial was judged statistically by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05. The regression coefficients were then used to make statistical calculation to generate contour maps from the regression models.

By applying a regression analysis to the experimental data it was possible to obtain a second degree polynomial model that explains the total variation of the amino peptidase activity:

\[ y = 538.14 + 24.79x_1 + 3.13x_2 + 37.79x_3 + 111.38x_4 - 24.36x_1^2 
+ 6.19x_1x_2 + 11.31x_1x_3 - 4.69x_1x_4 - 35.48x_2^2 - 14.06x_2x_3 
+ 1.94x_2x_4 - 37.78x_3^2 - 13.44x_3x_4 - 53.74x_4^2 \]
activity); $x_1$ is the wheat bran proportion; $x_2$ the water ration, $x_3$ is the raw material cooking time and $x_4$ is the koji making time.

Table 1 also presents the predicted data for amino peptidase activity (data shown in Table 1), in terms of the values of effects of the coefficients and $p$ (level of significance) of the variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ration were highly significant. The high significance of four factors on second-order interactions. The three factors and the second-order of all variables studied as well as their interactions. The three factors and the second-order of all four factors except water ratio and koji making time to the amino peptidase activity was higher. The surface response in Figure 2 illustrates that the interaction between wheat bran and water, wheat bran proportion 41% (w/w), water ratio 100% (v/w), raw material cooking time 21 min, koji making time 44 h, which are the optima values suggested by it. In this case, the coded settings of the tested variables were $x_1=0.51$, $x_2=0.04$, $x_3=0.38$ and $x_4=0.97$, respectively, the model predicting enzymatic activity recovery is 606 U/g. Experimentally, 598 U/g of enzymatic activity recovery was obtained, which confirmed the closeness of the model to the experimental results.

Table 2 gives the statistical analysis for the amino peptidase activity (data shown in Table 1), in terms of the values of effects of the coefficients and $p$ (level of significance) of the variables studied as well as their interactions. The three factors and the second-order of all four factors except water ratio and koji making time to the amino peptidase activity was higher. The surface response in Figure 2 illustrates that the interaction between wheat bran and water, wheat bran proportion 41% (w/w), water ratio 100% (v/w), raw material cooking time 21 min, koji making time 44 h, which are the optima values suggested by it. In this case, the coded settings of the tested variables were $x_1=0.51$, $x_2=0.04$, $x_3=0.38$ and $x_4=0.97$, respectively, the model predicting enzymatic activity recovery is 606 U/g. Experimentally, 598 U/g of enzymatic activity recovery was obtained, which confirmed the closeness of the model to the experimental results.

The interactions among these variables were shown in Figure1 to 6. Figures 1, 2 and 3 show the surface response obtained for the effects of wheat bran and water, wheat bran and cooking time, wheat bran and time to amino peptidase activity in koji making.

The interactions between these two effects did not cause a significant difference in the amino peptidase activity during koji making. It was found that for the proportion of wheat bran about 40%, the amino peptidase activity was higher. The surface response in Figure 2 illustrates that the interaction between wheat bran and cooking time was closest to the established $p$ value ($p=0.053$), indicating that the employ the cooking time 18-22 min, according with the wheat bran proportion 40%-43%, could increase the amino peptidase activity during koji making.

The interaction effects of water ratio and cooking time, water ratio and koji making time to the amino peptidase activity was calculated to be indicated that 99% of the variability of the model coefficient of determination of the model (R-squared = 0.9851) was calculated to be indicated that 99% of the variability in the response could be explained by the model.

Table 2. Statistical analysis of the process variables and their interactions for the amino peptidase activity.

<table>
<thead>
<tr>
<th>Variable Master model</th>
<th>F</th>
<th>t</th>
<th>Pr &gt; F</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1$</td>
<td>31.410</td>
<td>5.604</td>
<td>0.000</td>
<td>24.792</td>
</tr>
<tr>
<td>$X_2$</td>
<td>0.499</td>
<td>0.706</td>
<td>0.490</td>
<td>3.125</td>
</tr>
<tr>
<td>$X_3$</td>
<td>72.987</td>
<td>8.543</td>
<td>0.000</td>
<td>37.792</td>
</tr>
<tr>
<td>$X_4$</td>
<td>633.912</td>
<td>25.178</td>
<td>0.000</td>
<td>111.375</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>36.128</td>
<td>-6.011</td>
<td>0.000</td>
<td>-24.359</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>1.304</td>
<td>1.142</td>
<td>0.270</td>
<td>-4.687</td>
</tr>
<tr>
<td>$X_3^2$</td>
<td>76.666</td>
<td>-8.756</td>
<td>0.000</td>
<td>-35.484</td>
</tr>
<tr>
<td>$X_4^2$</td>
<td>6.373</td>
<td>-2.956</td>
<td>0.020</td>
<td>-14.062</td>
</tr>
<tr>
<td>$X_1X_2$</td>
<td>0.128</td>
<td>0.358</td>
<td>0.725</td>
<td>1.938</td>
</tr>
<tr>
<td>$X_1X_3$</td>
<td>96.736</td>
<td>-9.835</td>
<td>0.000</td>
<td>-37.775</td>
</tr>
<tr>
<td>$X_1X_4$</td>
<td>6.152</td>
<td>-2.480</td>
<td>0.025</td>
<td>-13.438</td>
</tr>
<tr>
<td>$X_2X_3$</td>
<td>175.807</td>
<td>-13.259</td>
<td>0.000</td>
<td>-53.734</td>
</tr>
</tbody>
</table>

*, Significant at 99% confidence level; **, significant at 95% confidence level.


g = \frac{y - \bar{y}}{s_y} = \frac{538.14 + 24.79x_1 + 37.79x_2 + 111.38x_3 - 24.36x_1^2 - 35.48x_2^2}{4} - 14.06x_1x_2 - 37.78x_1^2 - 13.44x_2x_3 - 53.74x_3^2 \tag{4}

Where $y$ is the predicted response (amino peptidase activity).
activity were shown in Figures 4 and 5. Figure 6 showed the cooking time, koji making time and the interaction effects on the activity of amino peptidase in koji making. The surface response in Figures 4 and 6 were closest.

Figure 1. Response surface showing the effect of water ratio and wheat bran proportion on amino peptidase activity.

Figure 2. Response surface showing the effect of cooking time and wheat bran proportion on amino peptidase activity.

to the established $p$ value ($p = 0.020, 0.025$), indicating that employed the cooking time 20-23 min, associated with the water ratio of 98%-105% and koji making time 42-46 h, significant increased the amino peptidase activity during koji making.

It is well known that the object of koji making is to produce protease and amylase (Yen-Yi et al., 2010). So enzyme activities of protease and $\alpha$-amylase were
assayed at the optimum condition of amino peptidase produced. The protease activity is 5543 U/g, the α-amylase activity is 43 U/g. It can meet the demand in soy sauce production.

Figure 3. Response surface showing the effect of time and wheat bran proportion on amino peptidase activity.

Fixed levels: water=100 %; cooking time=20 min

Figure 4. Response surface showing the effect of cooking time and water ratio on amino peptidase activity.

Fixed levels: wheat bran=40 %; time=42 h

The response surface methodology was successfully applied to perform the complete set of optimized variables values for the optimization of amino peptidase-biosynthesis by solid state fermented in making koji. The existence of interactions between the independent variables with the response was observed and established. The optimum parameter in koji making as following: wheat bran proportion (X1)41% (w/w), water
ration($X_2$) 100% (v/ w), raw material cooking time($X_3$) 21 min, koji making time($X_4$) 44 h. In the optimized condition the amino peptidase activity obtained was 598 U/g, which is 19% higher than comparison with the activity (495 U/g) obtained without optimization. By application of an experimental design approach, it can determination of the optimal parameter in the producer of koji making and can leads to an maximum level of amino peptidase activity. It will do beneficial to fermented Soy sauce.

REFERENCES


