

A Simple and Cost-Saving Approach To Optimize the Production of Subtilisin NAT by Submerged Cultivation of *Bacillus subtilis* Natto

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Subtilisin NAT, formerly designated nattokinase or subtilisin BSP, is a potent cardiovascular drug because of its strong fibrinolytic activity and safety. In this study, one *Bacillus subtilis* natto strain with high fibrinolytic activity was isolated. We further studied the optimal conditions for subtilisin NAT production by submerged cultivation and three variables/three levels of response surface methodology (RSM) using various inoculum densities, glucose concentrations, and defatted soybean concentrations as the three variables. According to the RSM analysis, while culturing by 2.93% defatted soybean, 1.75% glucose, and 4.00% inoculum density, we obtained an activity of 13.78 SU/mL. Processing the batch fermentation with this optimal condition, the activity reached 13.69 SU/mL, which is equal to 99.3% of the predicted value.

KEYWORDS: Subtilisin NAT; nattokinase; *Bacillus subtilis* natto; response surface methodology

INTRODUCTION

Natto is a traditional fermented food in Japan with a history of more than 1000 years. Its functions for health, including reducing blood cholesterol levels, decreasing blood pressure, and inhibiting osteoporosis, have made it a popular functional food worldwide (1). Accounts of cardiovascular diseases have become the leading cause of death in the Western world (2); many blood clot-dissolving agents, such as urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been utilized in clinical treatments for cardiovascular diseases. However, urokinase and t-PA are very expensive, and t-PA has a short half-life (3); streptokinase is a nonhuman protein and is immunogenic. The most severe defect of these blood clot-dissolving agents is the possibility of causing hemorrhagic side effect, which is fatal (4).

Subtilisin NAT (formerly designated nattokinase or subtilisin BSP), produced by *Bacillus subtilis* natto, is a serine protease (5) and is reported to have potent fibrinolytic activity (6). Besides in vitro tests of fibrinolytic activity, many in vivo studies had been reported. Sumi et al. (7) treated dogs with nattokinase by oral administration, and the fibrinolytic activity in plasma increased. Fujita et al. (8, 9) proved that subtilisin NAT could pass the rat intestinal tract and dissolve the chemically induced thrombosis. Suzuki et al. (10) found that dietary supplementation of natto suppressed intimal thickening and modulated the lysis of mural thrombi after endothelial injury in rat femoral artery. Yamashita et al. (11) also reported a similar effect of dietary *Bacillus natto* productive protein on in vivo endogenous thrombolysis.

Similar fibrinolytic enzyme-producing bacteria have also been isolated from Japanese shiokara (12), Korea chungkook-jang (13), and Chinese douchi (14). Nevertheless, it is still the most stable and economic way to obtain protein with fibrinolytic activity by *B. subtilis* natto. On the basis of its food origin, relatively strong fibrinolytic activity, stability in the gastrointestinal tract, and convenient oral administration, subtilisin NAT has advantages for commercially used medicine for preventative and prolonged effects.

Recently, statistical designs, including the Taguchi method and response surface methodology (RSM) (15, 16), have been employed to optimize enzyme production. Optimization through factorial design and response surface analysis is a common practice in biotechnology. Various research workers have applied this technique, especially for the optimization of culture conditions (17), such as lipase (18), xylanase (19), and monacolin K (20). In this study, we isolated *B. subtilis* natto strains from commercial natto products and optimized the culture conditions by RSM to find a simple way to produce subtilisin NAT with a low cost of cultivation. To our knowledge, this is the first paper using a cheap substrate to produce valuable subtilisin NAT.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions. *B. subtilis* natto was isolated from commercial natto collected in supermarkets. Two granules of natto were inoculated into a 50 mL Hinton flask with 10 mL of nutrient broth (Difco Laboratories Inc., Detroit, MI); after shaking flask cultivation for 24 h, single colonies were obtained by streaking plates. In this study, we also used various concentrations of defatted soybean combined with glucose as the medium. All shaking flask cultures in this study were maintained in an orbital shaker set at 150 rpm and 37 °C.

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Strain Screening. All strains isolated from commercial natto were further screened by fibrin-plate method (21). Thrombin from bovine plasma (Sigma, St. Louis, MO) solution of 0.5 mL (50 NIH U/mL) was added into 10 mL of bovine fibrinogen (Sigma) solution [5 mg/mL in borate saline buffer (0.01 M Na₂B₄O₇, 0.16 M H₃BO₃, and 0.04 M NaCl, pH 7.8)] in a Petri dish and kept at room temperature (25 °C) for 1 h for fibrin clots forming, after which the fibrin plate was ready. Ten microliters of the sample was dropped on the plate and incubated at 37 °C for 3 h. The relative fibrinolytic activity was determined according to the area of clear zones.

The commercial product that we used was manufactured by a Japanese brand "Okame Natto". In this study, we collected many kinds of products and screened the enzyme activity of each product because there would be more opportunity to find *B. subtilis* natto strain with higher activity. The product with the strongest activity was chosen. The strain that we isolated was then identified as *B. subtilis* ssp. *subtilis* by the Food Industry Research and Development Institute (Hsin-chu, Taiwan) according to the results of photomicrograph, 16S rDNA sequencing, and microorganism fatty acid identification system. The identification report number was 97ID017.

Fibrinolytic Activity Determination. The fibrinolytic activity was analyzed by chromogenic method (22). Synthetic substrate (S-2251), H-D-Val-Leu-Lys-pNA (Sigma), was used in fibrinolytic activity determination.

One unit of SU was defined as 1 nmol of *p*-nitroaniline released from substrate S2251 per minute per milliliter. Synthetic substrate (S-2251), H-D-Val-Leu-Lys-pNA (Sigma), was used in fibrinolytic activity determination. A 0.2 mL amount of Tris-HCl (0.1 M Tris-HCl and 0.2 M NaCl, pH 9.0) and 0.1 mL of sample were mixed and incubated at 37 °C for 2 min. A substrate solution of 0.2 mL (5 mM) was added into the mixture and incubated at 37 °C for 5 min. The reaction was stopped with 0.5 mL of trichloroacetate (0.2 M). After centrifugation for 5 min at 10000g, the supernatant was recovered and measured at 405 nm to get A₁. If the reaction was stopped before adding S-2251, then the A₄₀₅ was A₀. The pNA concentration was determined by the following equation:

$$\frac{\Delta A_{405}/\text{min}}{\epsilon \times 10^6 \times 0.1} = \frac{\Delta A_{405}/\text{min}}{9950 \times 10^6 \times 0.1} = 201 \times \Delta A_{405}$$

$$\Delta A_{405}: A_1 - A_0$$

where ΔA_{405} is $A_1 - A_0$ and ϵ is the extinction coefficient of pNA at 405 nm.

Design of Experiments and Model. RSM is a reliable and useful statistics methodology for investigating the optimal condition. RSM is usually used to investigate the effects of the condition factors on the changes of target product or markers through multiple views provided by a curve surface figure (23). This study used RSM to investigate optimum culture conditions including three factors: defatted soybean concentration, glucose concentration, and inoculum density.

Defatted soybean and glucose were the main nutrients for growing and, accordingly, were chosen as two variables. As for the third, cultivation time, rotation speed of the shaker, inoculum density, etc. might be chosen. However, we found that the cultivation time was not suitable because in different medium conditions, the activity would start to be driven high at similar time points. Also, our pretest showed that the rotation speed of the shaker was not important.

To identify the optimum condition, a Box-Behnken design (24) was selected. The crucial factors considered in this study included defatted soybean concentration (X_1), glucose concentration (X_2), and inoculum density (X_3). These factors, and the level at which the experiments were carried out, are shown in **Tables 1** and **2**. A total of 15 runs with central points were generated. The central point of the design arrangement used in this study was a defatted soybean concentration of 3.0%, a glucose concentration of 1.0%, and an inoculum density of 5.0%. Each run of experiments was carried out in a 500 mL Hinton flask.

RSM. The analysis of data was carried out using response surface regression (RSREG) in statistical analysis system (SAS, Cary, NC). A

Table 1. Process Variables and Levels in the Three Variables/Three Levels Response Surface Design

variable	symbol	coded variable level		
		-1	0	1
defatted soybean (%)	X_1	2.0	3.0	4.0
glucose (%)	X_2	0.5	1.0	1.5
inoculum density (%)	X_3	4.0	5.0	6.0

Table 2. Process Variables and Levels in the Three Variables/Three Levels Response Surface Central Composite Design Arrangement

runs	defatted soybean (%)	glucose (%)	inoculum density (%)
1	4.0 (1)	1.5 (1)	5.0 (0)
2	4.0 (1)	0.5 (-1)	5.0 (0)
3	2.0 (-1)	1.5 (1)	5.0 (0)
4	2.0 (-1)	0.5 (-1)	5.0 (0)
5	4.0 (1)	1.0 (0)	6.0 (1)
6	4.0 (1)	1.0 (0)	4.0 (-1)
7	2.0 (-1)	1.0 (0)	6.0 (1)
8	2.0 (-1)	1.0 (0)	4.0 (-1)
9	3.0 (0)	1.5 (1)	6.0 (1)
10	3.0 (0)	1.5 (1)	4.0 (-1)
11	3.0 (0)	0.5 (-1)	6.0 (1)
12	3.0 (0)	0.5 (-1)	4.0 (-1)
13	3.0 (0)	1.0 (0)	5.0 (0)
14	3.0 (0)	1.0 (0)	5.0 (0)
15	3.0 (0)	1.0 (0)	5.0 (0)

second-order model was employed to fit the data individually for the responses Y (fibrinolytic activity) by the general mode (25), with three factors, each coded factor was in the range of -1, 0, and +1. The second-order equation is as follows:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2$$

X_1 , X_2 , and X_3 are the three variables in the model, and this second-order equation was employed to fit the data individually for the responses Y . It means that this equation figures and simulates the results of various combinations of cultivation conditions. After 15 runs of central composite design arrangement, we can obtain the coefficients of this equation (A_0 , A_1 , A_2 , A_3 , A_{12} , etc.) by statistical software.

Batch Fermentation. Batch fermentation was carried out using a 500 mL Hinton flask. Cell cultures were first grown overnight and inoculated into 250 mL flasks containing fresh nutrient broth (Difco Laboratories Inc.). After 12 h, the seeding culture was transferred into a 500 mL Hinton flask to a final volume of 100 mL and maintained in an orbital shaker set at 150 rpm and 37 °C.

RESULTS

Strain Screening. Among 34 strains of *B. subtilis* natto, strain 12-1-2 harbored the largest clear zone (data not shown). This strain was further identified and confirmed by Food Industry Research and Development Institute (Hsin-chu, Taiwan).

Only the supernatant of fermented samples was detected in the fibrin plate test. Each sample would cause a clear zone on the plate, and the area was measured for relative comparison.

Confirmation of Subtilisin NAT. Nattokinase (subtilisin NAT) digested not only fibrin but also the plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251), which was more sensitive to the enzyme than other substrates tried (6). To confirm whether it was subtilisin NAT, the chromosomal DNA of the strain was extracted, and then, the DNA of subtilisin NAT was amplified by polymerase chain reaction (PCR). **Figure 1** was the result of electrophoresis, showing that its length was about 1100 bp, which was a correct length (26). The sequence was further

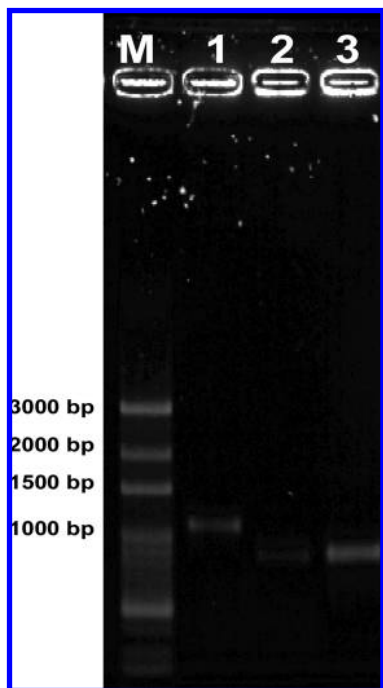


Figure 1. Molecular cloning of various lengths of *aprN* gene by PCR. Lane M, 100 bp DNA ladder; lane 1, PCR product of pro-NK; lane 2, PCR product of mature NK; and lane 3, PCR product of NK-histag.

confirmed by Mission Biotech (Taipei, Taiwan) and was completely the same as the *aprN* gene, which encodes subtilisin NAT.

Central Points of RSM. In RSM, the central points of each condition were studied at first. As shown in **Figure 2a**, while glucose concentration and inoculum density were set at 1.0 and 3.0%, respectively, 3.0% of defatted soybean would obtain the highest enzyme activity. In **Figure 2b**, both defatted soybean concentration and inoculum density were set at 3.0%; 1.0% of glucose would obtain the largest activity. In **Figure 2c**, the highest activity was obtained when the inoculum density was 5.0%, and concentrations of defatted soybean and glucose were set at 3.0 and 1.0%, respectively. Each best concentration of different conditions was chosen as central points.

Regression Equation, r^2 Value of Model. Data from 15 experiments were used to determine the regression equation in which the factors were obtained from regression analysis for the subtilisin NAT activity: fibrinolytic activity = $94.99 + 16.29X_1 - 18.84X_2 + 2.75X_3 - 28.65X_1X_2 + 0.78X_2X_3 + 12.95X_1^2 - 5.49X_2^2$. The variability in the responses was accounted for by the factor (r^2 value) for the model, and the data are given in **Table 3**. The r^2 value of fibrinolytic activity was 0.87. Also, the p value for the overall regression is significant at the 5% level, which indicates that the model is adequate in approximating the response surface of the experimental design.

Effects of Different Culture Conditions. As shown in **Figure 3**, in accordance with the regression equation, response surfaces were obtained by SPSS (SPSS Taiwan Co., Taipei, Taiwan) and Sigma Plot 8.0 (Aspire Software International, Ashburn, VA). The highest activity was situated on the lower response surface in which the inoculum density was 4.0%. Because the response surfaces were constituted by continuous responses of various conditions, we could not show all of the results on the plot. However, according to the mathematics

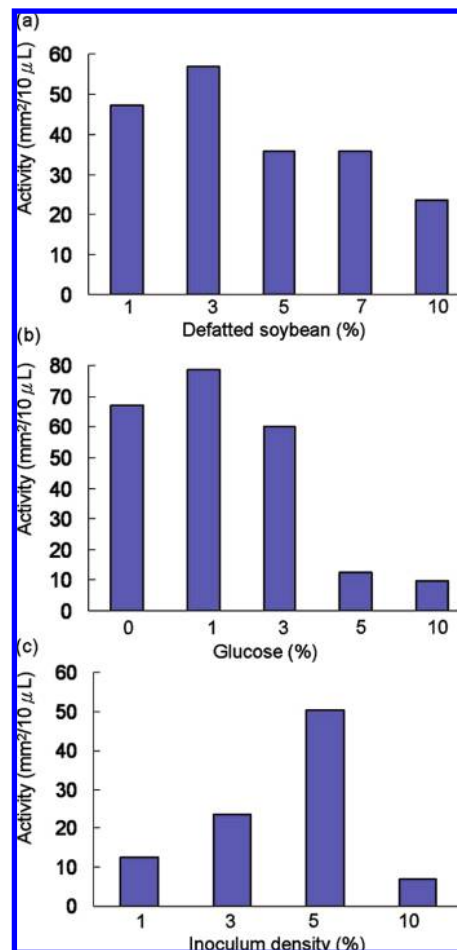


Figure 2. Effects of various factors in RSM. (a) Defatted soybean concentration, (b) glucose concentration, and (c) inoculum density.

Table 3. Analysis of Variances for the Activity of Subtilisin NAT with Various Culture Conditions

source	degrees of freedom	sum of square
		activity
regression	9	126.0
residual	5	19.0
lack of fit	3	17.0
pure error	2	2.0
variability (r^2)		0.87

operated by software, the optimal condition was 2.93% defatted soybean, 1.75% glucose, and 4.00% inoculum density, and the activity was expected to reach 13.78 SU/mL.

Optimal Batch Fermentation. The batch fermentation was performed by the optimum medium using a 500 mL Hinton flask. As shown in **Figure 4**, the log phase started from the sixth hour and stopped at the 12th hour, the beginning of the stationary phase. After 24 h, viable cells reached 2.88×10^9 CFU/mL. The enzyme activity appeared at the ninth hour and continuously increased before the 24th hour. After the 24th hour, it reached the highest activity at the 34th hour and sustained through the rest of the time of cultivation. The highest activity detected was 13.69 SU/mL, reaching 99.3% of predicted value.

DISCUSSION

Subtilisin NAT has been proved to have strong fibrinolytic activity. There are many advantages, including its food origin and safety, to apply subtilisin NAT to thrombosis therapy.

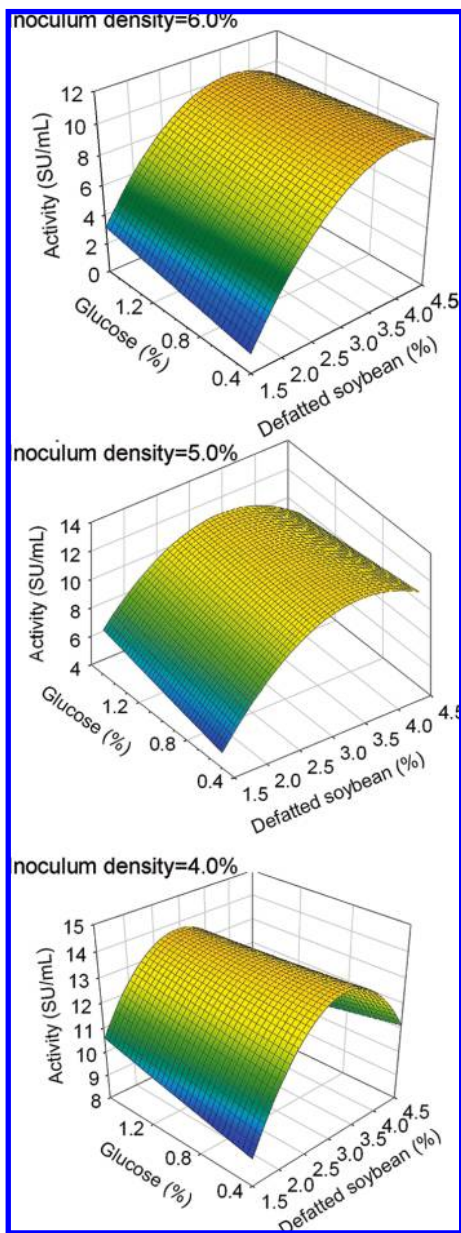


Figure 3. Response surfaces for the activity of subtilisin NAT produced by *B. subtilis* natto 12-1-2 submerged culture at various concentrations of defatted soybean, glucose, and inoculum density.

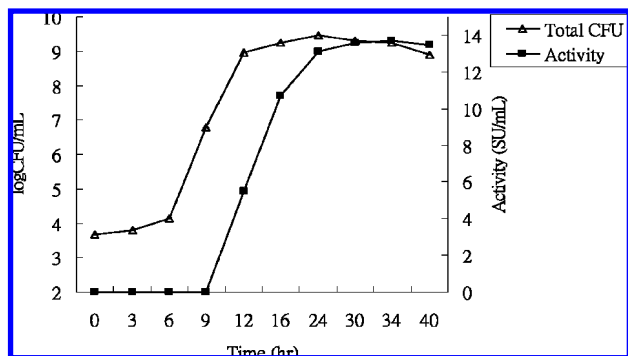


Figure 4. Time course for batch culture of *B. subtilis* natto 12-1-2 in a 500 mL Hinton flask with the optimal condition obtained by RSM. The cultural temperature was maintained at 37 °C, and the rotary shaker was set at 150 rpm.

Recently, many genetic engineering methods, for example, heterologous expression by *Escherichia coli* or *Spodoptera*

Table 4. Studies of Nattokinase Production^a

nattokinase-producing strains or cells	activity	ref
	U	
<i>B. subtilis</i> from thua nao	9.6 U/mL	(29)
recombinant <i>S. frugiperda</i> cells	60.0 U/mL	(28)
recombinant <i>E. coli</i>	260.0 U/mL	(30)
<i>B. subtilis</i> var. <i>natto</i>	3109.0 U/mg	(31)
	CU	
recombinant <i>B. subtilis</i>	71500.0 CU/mL	(32)
	SU	
<i>B. subtilis</i> ssp. <i>subtilis</i> 168	11.10 SU/mL	(33)
<i>B. subtilis</i> from natto	13.69 SU/mL	this study

^a U, activity detected using urokinase as a standard; CU, activity detected using α-casein as a substrate; and SU, activity detected using S-2251 as a substrate.

frugiperda cells, of producing subtilisin NAT have been reported (27, 28).

However, low expression efficiency of recombinant hosts, low yield, and instability of recombinant subtilisin NAT confine it to the application of industrial scale production. In this study, avoiding uncertainty of genetic engineering, we utilized *B. subtilis* natto and RSM to optimize the medium composed of defatted soybean, a byproduct in salad oil manufacturing, and glucose, a very common and cheap carbon source.

In our results of RSM, as shown in **Figure 3**, a higher inoculum density would cause the decrease of the enzyme activity, and decreasing defatted soybean concentration would cause the enzyme activity to drop dramatically. The activity would be decreased to less than 70% when the inoculum density reached 6.0%. Less seeding culture would generate more viable cells with a higher ability to produce the enzyme.

While under conditions of low defatted soybean concentration, the activity increased with glucose concentration, and this trend became more obvious in low inoculum density. However, under high defatted soybean concentrations, the glucose concentration had less of an effect on the activity. Consequently, defatted soybean played a more important role in the cultivation. In the optimal batch fermentation, *B. subtilis* natto grew stably in the medium and with fibrinolytic activity as expected.

As shown in **Table 4**, much research about subtilisin NAT production has been reported. Omura et al. (33) also reported correspondence results by adding glucose into soybean milk. In this study, we utilized defatted soybean to be the main origin of nutrition, using less runs of the experiment to optimize the culture condition.

The most important advantage is the low cost of defatted soybean and glucose. The costs per kilogram of nutrient broth (Difco Laboratories Inc.), glucose (Sigma), and defatted soybean were NT 6400, NT 320, and NT 30, respectively. Consequently, the cost of 1 L of the optimal medium (29.5 g of defatted soybean and 17.5 g of glucose) in this study is less than 13% of 1 L of nutrient broth (8 g/L). Defatted soybean is a suitable ingredient in the cultivation and production of *B. subtilis* natto and subtilisin NAT. To our knowledge, this paper is the first one using cheap substrates to produce valuable subtilisin NAT.

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