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Optimization Of The Possibility Synthetic Nattokinase In Soybean Substrates To Orientation Products Development

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Abstract: *Nattokinase is an enzyme with strong fibrinolytic activity that can be used for preventing thrombolytic diseases. In this study, we make survey the effect fermentation conditions to B.subtilis natto strain on the soybean substrate to enhance the nattokinase activity and optimization biosynthesis capabilities of nattokinase by Plackett-Burman experimental combined with response surface methodology RSM-CCD. The optimal results received nattokinase activity 136.6 FU/g. We also try to create dried products by freeze drying process in the conditions -80°C, 24 hours, 6-7 Pa. After that we examined moisture and nattokinase activity in these product. The results of experiments show that moisture was 4.3%, nattokinase activity was 515 FU/g. This research help expand application for creating soybeans powder products with hight nattokinase activity.*

Keywords: *Bacillus subtilis natto, RSM-CCD, Plackett-Burman, nattokinase, plasmin, fibrin, plasminogen, Natto*

I. Introduction

According to World Health Organization estimates that 17.3 million people died of thrombosis in 2008. Every year in the United States (USA) has about 6 million people with heart failure, of which there are about 1,5 million people with cardiovascular disease. The cause of more than 28% of deaths worldwide by thrombosis. Although the human body has several kinds of enzymes for creating thrombi, it uses only one "plasmin" for decomposing and dissolving thrombi. The properties of nattokinase closely resemble that of plasmin. Nattokinase, extracted from Natto, is a fibrinolytic enzyme that effectively breaksdown fibrin strands and the thrombi this fibrin holds together[5]. Nattokinase, therefore, undeniably promotes mechanisms of action that occur naturally. Once fibrin is degraded, clotting time is slowed. Nattokinase has been found to lyse (or breakdown) fibrin strands and plasmin substrates directly[4]. In the process of clot regulation, prokinase is converted into urokinase, a process that is enhanced by nattokinase. Breaking fibrin down into its degradation products is also enhanced, converting plasminogen to plasmin. Nattokinase increases tissue plasminogen activator also, enhancing fibrin breakdown and clotting reduction further. By reducing thrombus formation, nattokinase decelerates the progression of plaque formation and reverses evolving atherosclerotic lesions[10]. The aim of this study was to optimize the fermentation conditions to maximize nattokinase activity.

II. Materials And Methods

2.1 Materials

Microorganism and inoculum preparation: In this study, *Bacillus subtilis* natto was provided from Ho Chi Minh City University of Technology. The microorganism was grown on Nutrient Agar 37°C in 24h to prepare for next stage.

Chemicals: Crystal violet, fushin, lugol, phosphat buffer saline (PSB), fibrinogen, borate buffer (pH 8.5), thrombin , trichloroacetic.

- The analysis was performed at Biotechnology lab Ho Chi Minh City University of Technology and experimental procedure to create dried soybean powder products is performed at Ho Chi Minh City University of Food Industry

2.2 Methods

2.2.1 Microbiological Methods

Macroscopic observed protocol: Preparing Petri dishes with Nutrient Agar and the test tube containing 9ml distill water was sterile to diluting sample. Dilutting of the culture to obtain concentration 10^{-3} to 10^{-7}. Taking 0.1ml inoculate into Petri dishes and using to inoculating hook to can through the entire surface of the disk. Petri dishes were incubated at temperature 37°C for 1 day to form colonies. Observing and recording morphological, color and shape of colonies [18]

Observing microscopically by staining: Cell staining is a technique that can be used to better visualize cells and cell components under a microscope. By using different stains, one can preferentially stain certain cell components, such as a nucleus or a cell wall, or the entire cell. Most stains can be used on fixed, or non-living

cells, while only some can be used on living cells; some stains can be used on Gram (+) or Gram (-) microorganisms.[18]

Method of determining *B.subtilis* natto proliferation with time: *B.subtilis* natto was cultured on Nutrient Agar culture. After that, determine the number of bacterial cell by plate count method after 12 hours. Building growth curve by graphing the variation of biomass cells with time. Thereby determining the stage of development of microorganisms.[2]

2.2.2 The Plackett-Burman design and response surface methodology RSM-CCD

The Plackett-Burman design: The aim of this study was to obtain the optimal operation conditions of the fermentation process in order to achieve maximum yield of nattokinase, which was determined by the nattokinase activity assay. The higher enzyme activity obtained from the fermentation represented the higher yield of nattokinase activity. After the fermentation process was carefully reviewed, four factors appeared to be very essential to the enzyme productivity. Screening the significant variables based on the preliminary study, we chose the following four variables as the experimental variables: Bacteria inoculate (X1), time (X2), pH (X3) and temperature (X4). Each variable had two levels to be examined a high (+1) and low level (−1). The high and low levels selected represented the extremes of normal operating ranges. Table 1 shows the variables and levels in detail. The screening, experimental design. Design – expert 10.0 [3][1]

Table 1. The factor using on Plackett – Burman

Code of factors	**Name of factors**	**Limit of research**	**Values of factors**	
			Low	**Hight**
			-1	**1**
X1	Bacteria inoculate (logCFU/g)	5-7	5	7
X2	Time (hour)	24-48	24	48
X3	pH	7-8	7	8
X4	Temperature (°C)	32-42	32	42

Optimization by response surface methodology: Response surface methodology is an empirical modeling technique used to evaluate the relationship between a set of the controlled experimental variables and the measured responses. A prior knowledge and understanding of the process and process variables under investigation are necessary to create a more realistic model. Based on the results of variable screening, the model examined the experimental variables required for optimal nattokinase activity using the Plackett-Burman design and response surface methodology.

In developing the regression model, the experimental variables were coded according to the following equation:

$$X_{i=} \frac{X_i - X_0}{\Delta Xi}$$

where Xi is the coded value of the variable Xi, X0 is the value of Xi at the center point, and Xi is the step change value.The range and the levels of the experimental variables investigated in this study are shown in table 1 the Plackett - Burman design. The central composite design (CCD), which is widely used in practice because of its efficiency with respect to the number of runs required [12], is employed for fitting a second-order response surface model. Generally, a CCD for k factors requires 2k factorial runs, 2k axial runs and at least one center point (typically, three to five center points are used). From the step of factor identification, there are three important factors (i.e., Factors C, D and G) that may significantly affect the enzyme productivity. Table 2 shows the level planning of the CCD with each factor containing five levels (i.e., -α, -1, 0, +1 and +α). Fermented temperature was selected as the center level for this factor.[1][12]

Table 2. The factor using on RSM – CCD

Code of factors	***Name of factors***	***Limit of research***	***Value of factor***				
			-α	***-1***	***0***	***1***	***α***
x_1	Bacteria inoculate (LogCFU/g)	4.32-7.68	4.32	5	6	7	7.68
x_2	Time (hour)	15.82-56.18	15.82	24	36	48	56.18
x_3	pH	6.66-8.34	6.66	7	7.5	8	8.34

Once the experiments were performed, the regression model was constructed by fitting the experimental results with a second order polynomial. The optimal conditions of selected variables were found using the regression model and by analysis of the response surface plots.
Response functions are selected nattokinase activity (FU/g). Modeling is represented by a equation quadratic:
$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_1x_1^2 + b_2x_2^2 + b_3x_3^2$
Where Y is nattokinase activity (FU/g); x_1, x_2, x_3 respectively bacteria inoculate (logCFU), time (hour), pH ; b_1, b_2, b_3 is the coefficient level 1; b_{12},b_{13}, b_{23} is the interaction coefficients of each pair of elements; x_1, x_2, x_3, x_1x_2, x_1x_3, x_2x_3, x_1^2,x_2^2, x_3^2 is the independent variable. [11]

2.2.3 Nattokinase activity measurement
From fermentation broth dilute ratio of 1:1 with distill water to 50ml centrifuge tubes. The *B.subtilis* natto cells were harvested from the fermentation media via centrifugation (4,000 rpm) for 20 min at 4°C and then resuspended in Phosphate Buffered Saline. *B.subtilis* natto lysis was carried out by sonication on ice (6 times with 1-min intervals, at the 50W). The sonicated cell suspension was centrifuged at 4,000 rpm for 15 min at 4°C. After that collection 1ml supernatant centrifuged at 15,000 rpm for 15 min at 4°C to remove cellular debris, enzyme solution was used for activity assay [16].
Fibrinolytic enzyme activity was determined by measuring the hydrolysis ability on fibrin according to [17] with some modifications. To 1.4 mL of 50 mM sodium borate buffer (pH8.5), a 0.4 mL of 0.72% (w/v) fibrinogen solution was added and kept at 37°C for 5 min. To there sulting fibrinogen solution was then added 0.1 mL of thrombin (20U/mL) and kept at 37°C for another 10 min. After the addition of 0.l mL of enzyme, the proteolytic reaction was performed at 37°C for 60 min. After the reaction was ceased by adding 2 mL of 0.2 M trichloroacetic acid (TCA), samples were kept at ambient temperature for 20 min and then centrifuged at 14000 rpm for 5 min. One unit of fibrinolytic activity was defined as the amount of enzyme that caused an increase of 0.01in the absorbance at 275 nm within 60 min reaction at 37°C.[1]

2.2.4Statistical and analysis methods
Data collected from the experiment were treated and analyzed by statistical software Statgraphics centurion XVII, excell 2007. Plackett–Burman design and response surface methodology RSM-CCD design by Design expert 10.0

III. Results And Discussion

After diluted samples and cultured on Nutrient Agar, incubated 24 hours, examined the morphological characteristics of *B.subtilis* natto with recorded the results: Colony have circular sharp, dry surfaces, irregular margin looks like serrated, wrinkled surface. Continue to Gram staining and observing by microscope to examine the microscopic characteristics. [11][12] Gram staining and observing by microscope we found bacteria forms follows as: rod-shaped, violet color, short, small, round head. This is similar with the theoretical basisrod-shaped, oval spores, Gram-positive is approximately 0.5-0.8µm x 1.5-3.0µm. The colonies are round, their edges are irregular jagged, their diameters are from 3 to 5 mm, and they have light yellow, dark heart [2][3].
From the results of examining macroscopically and microscopically, we affirm thoroughbred *B.subtilis* natto. This strain will be subculture to keep and use for the next experiment.

3.1 Effect of fermentation conditions to nattokinase activity
The effect of bacteria inoculate *B.subtilis* natto to nattokinase activity: In this experiment bacteria inoculate surveyed from 10^4 CFU/g to 10^7 CFU/g. The survey results nattokinase activity after fermentation showed the highest activity is 10^6 CFU/g and 10^7 CFU/g, respectively 113.23 FU/g and 113.44 FU/g the difference do not statistically significant. Bacteria inoculate 10^6 CFU/g corresponding to 18h growth biomass level 1 and bacteria inoculate 10^7 CFU/g corresponding to 20h growth biomass level 1. Thus to reduce the time period of proliferation of breeding level 1 while ensuring bacteria inoculate to high enzyme activity, we chose bacteria inoculate 10^6 CFU/g.
The results effect of fermentation time to nattokinase activity: This survey was carried out in the time from 12h to 48h. Survey results enzymatic activities after the fermentation with time showed that the highest nattokinase activity at the time 36h and 48h fermented with nattokinase activity respectively 118.43 FU/g and 118.33 FU/g and differences do not statistical significance. From the results above survey to obtain fermented products with the highest enzyme activity and reduce the fermented time of the production process we selected fermentation time is 36h. The results are consistent with previous studies of Dia-Shin wanget al "Optimization of nattokinase production conduction using response surface methodology" with the optimum time is 37.0817 hour. [1]
The results effect of pH to nattokinase activity: In this experiment we survey pH from 5.5 to 8. The survey results from 5.5 to 7.5 corresponding enzyme activity increases with pH increase and decrease in pH 8. At pH 7

and pH 7.5 is the highest and the difference do not statistically significant. According to the survey results to ensure high enzyme activity, we can perform fermentation at pH 7 or 7.5. The result are consistent with previous studies of Peddapalli Siva Rasagnya1 And MeenaVangalapati "Studies on Optimization of Process Parameters for Nattokinase Production by *Bacillus subtilis* NCIM 2724 and Purification by Liquid-Liquid Extraction"[2]
The results effect of temperature to nattokinase activity: Through the survey above we know the highest nattokinase enzyme activity when fermented at a temperature of 37°C with nattokinase activity is 119.92 FU/g.

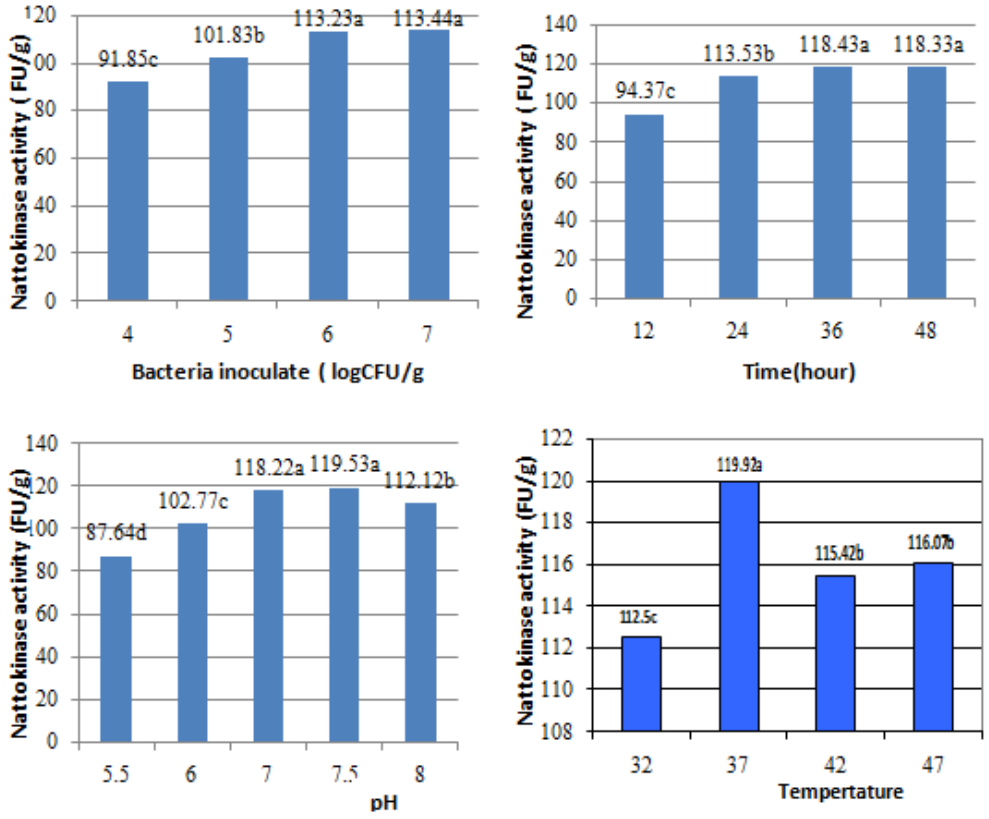

Fig1. Effect of fermentation conditions to nattokinase activity

3.2 Plackett–Burman design

The model F-value of 21151.67 implies the model is significant. There is only a 0.54% chance that an F-value this large could occur due to noise. Values of "Prob>F" less than 0.0500 indicate model terms are significant. In this case x1, x2, x3 are significant model terms, x4 are not significant. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 387.571 indicates an adequate signal. This model can be used to navigate the design space

Table 3. The results of Plackett-Burman design

Factor	Stdized effect	F-value	P-value
Model	1016.34	21151.67	0.0054
x1- bacteria inoculate	1.43	78.84	0.0714
x2-Time	45.82	80656.11	0.0022
x3-Ph	20.51	16166.34	0.005
x4-Temperature	-9.96	3859.06	0.0103

From table 3 and Fig 2 we can see fermentation time is the biggest effecting to nattokinase activity, followed by pH, bacteria inoculate. In the experiment, the fermentation temperature had very low effect and do not statistically significant.

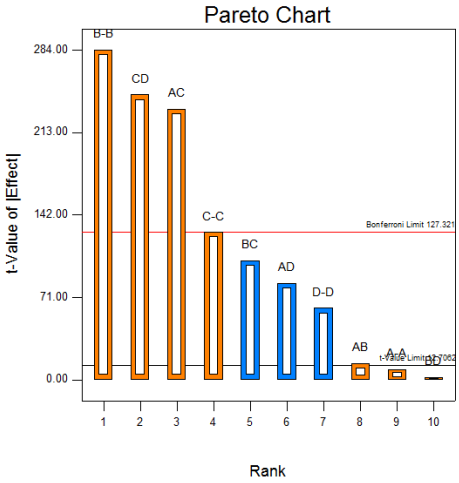

Fig 2. The level influence of the factors to nattokinase activity

From the results of this analysis, we are selected factors for the survey response surface methodology RSM-CCD design include time, pH and bacteria inoculate. Temperature is kept at the center 37°C.

3.3 Obtimization response surface methodology RSM-CCD design

The Model F-value of 36.99 implies the model is significant. There is only a 0.01% chance that an F-value this large this large could occur due to noise. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case x_1, x_2, x_3 are significant model terms. The "Pred R-Squared" of 0.7668 is in reasonable agreement with the "Adj R-Squared" of 0.9446, difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This ratio of 18.799 indicates an adequate signal. This model can be used to navigate the design space.

Table 4. The result Anova analysis of RSM-CCD

Factor	Coefficient estimate	Standard error	F-value	P-value
Model		2.76	36.99	<0.0001
x_1- Bacteria inoculate	7.56	1.83	16.98(**)	0.0021
x_2-Time	9.39	1.83	26.23(***)	0.0004
x_3-pH	6.23	1.83	11.53(**)	0.0068
x_1*x_2	-22.97	2.4	91.91	<0.0001
x_1*x_3	5.72	2.4	5.7(**)	0.0382
x_2*x_3	6.82	2.4	8.09(**)	0.0174
x_1^2	-17.51	1.78	96.2	<0.0001
x_2^2	-13.72	1.78	59.11	<0.0001
x_3^2	-12.62	1.78	50.01	<0.0001
R-Squared	0,9708			
AdjR-Squared	0,9446			
PredR-Squared	0,7668			
Adeqpricision	18.799			

Fig 3 showed that interaction between bacteria inoculate and time to nattokinase activity focus area near the center corresponds 10^6 CFU/g and 37h. This result shows that the selection of the parameters of the experiment is right for bacteria inoculate and time

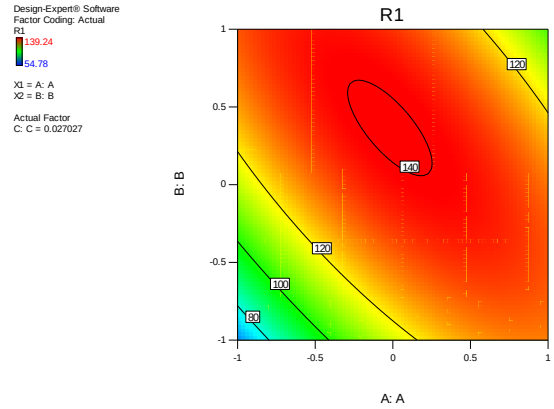

Fig 3. The interaction between bacteria inoculate and time

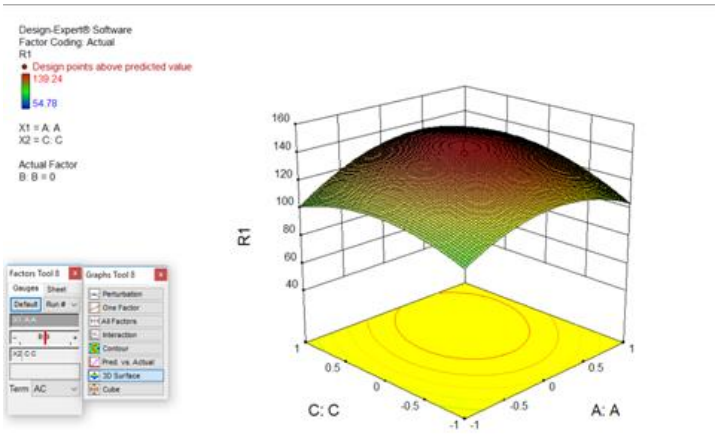

Fig 4. Plots of the activity response

Regression analysis gave the following second-order regression model:

$Y = 138.76 + 7.56\ x_1 + 9.39\ x_2 + 6.23\ x_3 - 22.97\ x_1x_2 + 5.72\ x_1x_3 + 6.82\ x_2x_3 - 17.51\ x_1^2 - 13.72\ x_2^2 - 12.62\ x_3^2$

Using RSM-CCD design allows us to predict nattokinase activity at 141.92 FU/g with bacteria inoculate 10^6 CFU/g, time 41h, pH 7. The parameters of the prediction modeling used to design the experiments with repeated 3 times, the results shown in the table 5 shows results 136.6 FU/g higher in all prior experimental treatments.

Table 5. There sults of experimental verify follow response surface methodology RSM-CCD

Bacteria inoculate (logCFU/g)	**Time (hour)**	**pH**	**Prediction nattokinase activity (FU/g)**	**Experiment nattokinase activity (FU/g)**	**The rate similer of experiment with theoretical (%)**
6	41	7.7	141.92	136.6	96.25

IV. Conclusions

From these studies we have conclusions following as:

Through the survey, the effects of fermentation conditions on nattokinase activity for best results in bacteria inoculateis10^6 CFU/g, fermentation time of 36 hours, fermentation pH 7.5, fermentation temperature 37^0C.

The Plackett-Burman design and response surface methodology RSM-CCD used to optimize the fermentation conditions for best results with optimal nattokinase activity 136.6 FU/g after fermentation conditions: Bacteria inoculateis10^6 CFU/g, fermentation time 41hours, fermentation pH 7.7

Initial trial of dried product soybean powder rich nattokinase activity, freeze drying in drying conditions: -80°C, 6-7 Pa, 24 hour. The dried product have nattokinase activity 515 FU/g, 4.3% moisture.

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