International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 13 No. 3 Nov. 2015, pp. 663-668

http://www.ijias.issr-journals.org/

DETERMINATION THE OPTIMUM FERMENTATION IN OBTAINING NATTOKINASE BY *BACILLUS SUBTILIS* NATTO

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ABSTRACT: Nattokinase is able to hydrolyze fibri oriented fibers, reducing blood pressure to the arteries and heart valves. People use nattokinase enzyme through ingestion by natto food or functional food to dissolve blood clots. In fermentation by *Bacillus subtilis* natto, the bacterium can generate this enzyme. The optimized culture medium has 6 factors such as glucose, soybean peptone, K_2HPO_4, $MgSO_4$, NaCl, $CaCl_2$ and the rate of these in medium is identified by response furface methodology (RSM) and central composite design (CCD). The highest nattokinase yield was 69.3 ± 0.2 FU/mL of substrate in optimized medium after 20 hours of fermentation in 37^0C and pH 7.5.

KEYWORDS: extracellular proteases, Nattokinase activity, optimized experiments, Plackett – Burman, response furface methodology, central composite design.

1 INTRODUCTION

Nattokinase is an essential enzyme dissolving blood clots, due to the ability to hydrolyze fibri oriented fibers, reducing blood pressure to the arteries and heart valves. Therefore, the heart rhythm is improved and the phenomenon stroke and stroke are prevented. Nattokinase containing products mainly derived from the fermentation of *Bacillus subtilis* natto bacteria on semi-solid medium with ripe soybean. Its fermentation creates some byproducts that they are capable of helping the metabolism and absorption of the stomach. Furthermore, Vitamin K in soybean involved in the clotting process and improved the calcium absorption. As the result, people use whole products from the fermentation on semi-solid medium like food. On the other hand, nattokinase enzyme is used in specific treatment, the important requirements are the highest purity. The clarity enzyme production is easier from liquid medium than that is from semi-solid culture [1-3].

The fermented medium factors are D-glucose, soybean peptone, K_2HPO_4, $MgSO_4.7H_2O$, NaCl, $CaCl_2$. Most bacteria need carbon source to build the cell structure, they can use many different soures such as D-glucose, sucrose, maltose, fructose, dextrin, enthanol, acid acetic …. D-glucose is the most important carbon source in which. *Bacillus subtilis* use D-glucose for building the cell formation and take part in the metabolism of substances. The nitrogen source which the bacteria use easily is organic nitrogen sources. The studied bacterium needs soybean peptone to improve the nattokinase enzyme metabolism. Moreover, $CaCl_2$ is the aroused factor in nattokinase enzyme metabolism of *Bacillus subtilis* natto. The other factors involve the biochemical reactions in cell of bacteria. The influence of these factors depends on their concentration in the cultural medium. If they are too high or too low, they become inhibitors the development of cell and enzyme biosynthesis. We must to optimize the appropriate concentration of them to increase performance for the enzyme production [4-6].

A bacterium that was isolated from Vietnamese natto food, is named *Bacillus subtilis* natto. The bacterium that was employed to study had the initial nattokinase yield of 23.583 ± 1.539 FU/mL of substrate in NB medium. Various culture-independent methods have been developed; in particular, methods using the variable and conserved regions of the 16S rRNA have proved successful in characterizing the gut microbiota. Sequencing of 16S rRNA genes has revealed that microbial diversity in the gut is far more extensive than previously described from studies of cultured microorganisms alone [7].

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The relationship between the factors which in fermented medium and nattokinase activity which in broth is identified by the optimized experiments. As well as the paper is claimed that the most suitable values of each factors which in fermented medium to be obtained the highest nattokinase activity.

2 MATERIALS AND METHODS

2.1 MATERIALS AND CULTURAL MEDIUM

The micro-organism specie: *Bacillus subtilis* was isolated from Vietnamese natto-food

The cultural medium: *Bacillus subtilis* natto grows in the nutrition broth (NB), pH 7.5, the temperature at 37^{o}C, the agitation rate of 150 rpm. After 20 fermentation hours, the cell rate in the broth was 50 billion colony-forming units.

The fermentation medium (g/L): glucose (1.25 – 10.00), soybean peptone (5 -15), K_2HPO_4 (1.25 – 3.00), $MgSO_4.7H_2O$ (0.25 – 1.50), NaCl (2.5 – 7.5) and $CaCl_2$ (0.05 – 0.40) were employed in the range studied.

The fermentation condition: temperature 37^{o}C, initial pH in medium 7.5 ± 0.3, adjusted by NaOH 1N solution.

2.2 EXPERIMENT DESIGNS

2.2.1 SCREENING THE FERMENTATION MEDIUM FOR PRODUCTION OF NATTOKINASE ENZYME BY *BACILLUS SUBTILIS* NATTO

Bacilus subtilis natto was incubated in NB medium. After 20 inoculum-hours, the number of the cells was checked and counted as well as added approximately 5 billion colony-forming units per milliter of fermentation medium.

Six variables were examined in the Plackett-Burman matrix with different 12-runs. We determined the Nattokinase acitivity for each validation formula and analyzed the factors that affect the Nattokinase activity by *Bacillus subtilis* natto. The main factors in experiments had p-value < 0.05. The chosen factors were designed to determine the relative between those and the response.

2.2.2 OPTIMIZING THE FERMENTATION MEDIUM FOR PRODUCTION OF NATTOKINASE ENZYME BY *BACILLUS SUBTILIS* NATTO

With the selected factors from the screening experiments, we carried out the initial experiments with the original values (+1, -1). Based on “Lack-of-fit” test, we determine the relationship between the chosen factors and response. After analyzing the initial experiments, we determined whether the factors having great impacts on the high regression equation suitably or not. If the regression is the linear function, we design the steepest experiments to identify the suitable range of factors.

Based on that, we conducted the experiments for response surface methodology having the central composite designs (RSM-CCD) and determined function of the polynomial regression accurately to describe relations between the enzyme activity in broth and medium factors.

The results of response optimizer that were simulated by software were measured by experiments to determine the highest actual nattokinase activity in broth.

2.3 THE ANALYZING METHOD

ANALYZING NATTOKINASE ACTIVITY IN BROTH

Nattokinase activity was determined by the ability to hydrolyze fibrin fibers. *Bacillus subtilis* natto was stopped the fermentation after 20 hours and broth was centrifuged at 13 000 rpm for 20 minutes and obtained the supernatant to determine nattokinase activity.

Tris–HCl (50 mM, pH 7.5) of 1.3 mL and 0.4 mL of 0.72% (w/v) fibrinogen solution were taken in vials and kept in water bath (37^{o}C) for 5 minutes. Then 0.1 mL thrombin (20 U/mL) was added and kept in water bath (37^{o}C) for 10 minutes. To this clot, 0.l mL of enzyme was added. After incubation (37^{o}C, 60 minutes), 2 mL of 0.2 M trichloroacetic acid (TCA) was added. Vials were kept 20 minutes and centrifuged at 3000 x g for 5 minutes. One unit enzyme activity is defined as the amount enzyme required to produce an increase in absorbance equal to 0.01 in 60 minutes at 280 nm [4].

Analyzing data and identifying the regression were used by Minitab 17.

3 RESULTS AND DISCUSSION

3.1 SCREENING MAIN EFFECTIVE FACTORS OF THE NATTOKINASE ENZYME PRODUCTION BY *BACILLUS SUBTILIS*

Bacillus subtilis natto grows and produces nattokinase enzyme on cultural medium that contained by D-glucose, soybean peptone and amount of multi-minerals and micronutrients such as KH_2PO_4, $MgSO_4$, NaCl and $CaCl_2$.

In the range of these factors in cultural medium, there were two factors that had a significant effect to biomass production like soybean peptone and $CaCl_2$, however, $CaCl_2$ hadnot been a significant effect ($p > 0.05$). In those ranges of factors in cultural medium that used to produce nattokinase enzyme, there were 5 factors that had a significant effect to the process including glucose (x_1), soybean peptone (x_2), K_2HPO_4, $MgSO_4$ and NaCl.

Table 1. The runs in Plackett-Burman matrix of screening experiment

Run	X_1	X_2	X_3	X_4	X_5	X_6	Response, (Y, FU/mL)
1	-1	-1	-1	+1	+1	+1	6.32
2	+1	+1	-1	+1	+1	-1	17.58
3	-1	+1	+1	+1	-1	+1	30.18
4	+1	-1	+1	-1	-1	-1	39.05
5	+1	+1	+1	-1	+1	+1	37.81
6	-1	-1	+1	+1	+1	-1	7.92
7	-1	+1	-1	-1	-1	+1	35.62
8	-1	-1	-1	-1	-1	-1	25.71
9	+1	-1	+1	+1	-1	+1	32.14
10	+1	-1	-1	-1	+1	+1	18.09
11	-1	+1	+1	-1	+1	-1	21.35
12	+1	+1	-1	+1	-1	-1	37.07

Table 2. The factors in Plackett-Burman matrix and its effects on nattokinase enzyme production by Bacillus subtilis natto

No.	Name of factors (g/L)	Symbols	The range of factors			The effect of factors	F-value	p-value	T-value
			Below (-1)	Centre (0)	Above (+1)				
1	Glucose	X_1	1.250	5.625	10.000	1.041	26.720	0.004	5.170
2	Soybean peptone	X_2	5.000	10.000	15.000	+0.840	22.720	0.005	4.770
3	K_2HPO_4	X_3	1.250	2.125	3.000	+2.670	7.050	0.045	2.650
4	$MgSO_4.7H_2O$	X_4	0.250	0.875	1.500	-6.190	19.280	0.007	-4.390
5	NaCl	X_5	2.500	5.000	7.500	-3.023	73.620	0.000	-8.580
6	$CaCl_2$	X_6	0.050	2.025	0.400	+5.470	1.180	0.327	1.090
DF = 6		R-sq = 96.79%							

Regression Equation in screening experiments:

$$\textbf{Response, Y (FU/mL)} = 25.11 + 1.041\,X_1 + 0.840\,X_2 + 2.67\,X_3 - 6.19\,X_4 - 3.023\,X_5 + 5.47\,X_6$$

In the above equation, y (g/L) symbols for nattokins activity (FU/mL), x_1 (g/L) symbols for glucose, x_2 (g/L) symbols for added soybean peptone, x_3 (g/L) symboys for K_2HPO_4, x_4 (g/L) symboys for $MgSO_4$ and x (g/L) symbols for sodium chlorua.

In the tested screening experiments, there were 5 factors such as glucose (x_1), soybean peptone (x_2), K_2HPO_4 (x_3), $MgSO_4.7H_2O$ (x_4) and sodium clorua (x_5) in the fermented medium that had leaded to the nattokinase activity. In *Bacillus subtilis* natto which we studied, it was different from concentration of cultural medium factors between the fermentation process in biomass production and nattokinase enzyme production [7].

3.2 OPTIMIZING FERMENTATION MEDIUM FOR NATTOKINASE ENZYME PRODUCTION BY *BACILLUS SUBTILIS* NATTO

3.2.1 INITIAL EXPERIMENTS

We went on conducting 13 experiments, 8 of them are (-1,1) ones and 5 are the central ones. ANOVA was carried out to statistically analyze the correlation of nattokinase activity to the five selected factors. The p-value of Lack-of-fit test was lower than 0.005 (p = 0.014) and R-sq was 97.92%.

Table 3. The runs in initial experiment and the responses (nattokinase activity)

Run	CenterPt	X_1	X_2	X_3	X_4	X_5	Response, (Y, FU/mL)
1	0	0	0	0	0	0	57.65
2	1	-1	-1	1	1	-1	29.97
3	1	1	-1	-1	-1	-1	51.43
4	0	0	0	0	0	0	55.82
5	0	0	0	0	0	0	56.18
6	1	-1	-1	-1	1	1	15.32
7	1	1	1	-1	1	-1	20.02
8	1	-1	1	-1	-1	1	20.95
9	1	1	1	1	1	1	23.85
10	0	0	0	0	0	0	54.91
11	0	0	0	0	0	0	55.65
12	1	1	-1	1	-1	1	40.61
13	1	-1	1	1	-1	-1	41.2
Rsq = 97.92%		p-value of “Lack-of-Fit” is 0.004					

Regression Equation in initial experiments:

$$\textbf{Response, Y (FU/mL)} = 47.050 + 0.813\,X_1 - 0.783\,X_2 + 3.990\,X_3 - 13.010\,X_4 - 2.095\,X_5$$

In the above equation, y (g/L) nattokins activity (FU/mL), x_1 (g/L) glucose, x_2 (g/L) added soybean peptone, x_3 (g/L) K_2HPO_4, x_4 (g/L) $MgSO_4$ and x (g/L) sodium chlorua.

3.2.2 RSM-CCDESIGNS

This means that the arrangement of the five significant effect factors is near the extreme of the aimed function and the polynomial regression between five selected factors and response could be given in the function of the poly-nominal regression. At this, the highest yield of all experiments was obtained. To establish the right relationship between the factors, we analyzed the 33 RMS-CCD experiments (shown in Table 4). It was given shown in the table that nattokinase activity (FU/mL) in the broth ranged from 10.21 to 57.65.

Regression Equation of Response:

$$\textbf{Response, Y}\left(\frac{\textbf{FU}}{\textbf{mL}}\right) = 27.6 + 19.9x_1 - 4.3x_2 - 35.6x_3 - 4.3x_4 + 10.5\,x_5 - 1.1\,x_1^2 + 11.48x_3^2 - 2.5x_4^2 - 1.5x_5^2 - 1.7x_1x_3 + 3.15x_3x_4$$

In the above equation, y (g/L) nattokins activity (FU/mL), x_1 (g/L) glucose, x_2 (g/L) added soybean peptone, x_3 (g/L) K_2HPO_4, x_4 (g/L) $MgSO_4$ and x_5 (g/L) sodium chlorua.

The response (nattokinase activity) is achieved at the bottom value when glucose (x_1) and sodium chlorua are increased while soybean peptone and K_2HPO_4 as well as $MgSO_4$ are dropped. KH_2PO_4 is the largest effect of all factors (with constant is – 35.6), the second highest effect is glucose (with constant is +19.9), following sodium chlorua (with constant is + 10.5). In the interactive effect between the factors, KH_2PO_4 and $MgSO_4$ have the largest relationship of whole factors.

Table 4. The runs in CCD-RSM experiments and the responses (nattokinase activity)

RunOrder	PtType	X1	X2	X3	X4	X5	Response, (Y, FU/mL)
1	1	-1	1	-1	-1	-1	23.15
2	0	0	0	0	0	0	57.65
3	1	1	1	1	1	1	23.85
4	1	-1	-1	-1	1	-1	15.94
5	-1	1	0	0	0	0	28.22
6	1	1	1	-1	1	-1	20.02
7	1	1	1	1	-1	-1	22.54
8	0	0	0	0	0	0	55.82
9	1	-1	-1	-1	-1	1	10.21
10	1	-1	1	-1	1	1	20.15
11	-1	0	0	0	-1	0	41.87
12	0	0	0	0	0	0	56.18
13	-1	0	1	0	0	0	49.26
14	1	1	-1	1	1	-1	43.54
15	1	1	-1	1	-1	1	42.98
16	1	-1	-1	1	-1	-1	43.07
17	-1	-1	0	0	0	0	11.21
18	1	1	-1	-1	1	1	31.27
19	-1	0	0	0	0	1	23.15
20	1	-1	-1	1	1	1	31.52
21	-1	0	0	0	1	0	36.25
22	-1	0	0	0	0	-1	38.75
23	-1	0	0	1	0	0	48.77
24	-1	0	-1	0	0	0	41.74
25	-1	0	0	-1	0	0	48.86
26	1	-1	1	1	-1	1	43.57
27	1	-1	1	1	1	-1	41.2
28	0	0	0	0	0	0	54.91
29	0	0	0	0	0	0	55.65
30	0	0	0	0	0	0	56.042
31	1	1	-1	-1	-1	-1	55.55
32	1	1	1	-1	-1	1	38.09
Rsq= 89.98%							

In this response, we used the Minitab to identify the extreme values of nattokinase activity. Muliple response is predicted is 70.5 FU/mL when x_1, x_2, x_3, x_4, x_5 are 6.1; 5.0; 3.0; 0,25 and 4,27 respectively. The fact value of response is 69.3 ± 0.2 FU/mL. The result is given that is twice as nattokinase activity as the initial test [7].

Chemoheterotrophic bacteria use a few central metabolic pathways for carbon catabolism and energy production as well as for the generation of the main precursors for anabolic reactions. Bacteria use all sources of carbon like energy resources in central pathways. In *Bacillus subtilis*, the presence of glucose is analyzed the role of the pleiotropic transcriptional regulator CcpA. In contrast, induction by glucose seems to be mediated by a variety of different mechanisms. In the presence of glucose, the genes encoding glycolytic enzymes are induced. Moreover, the genes responsible for the production of acetate from pyruvate with a concomitant substrate-level phosphorylation are induced by glucose. In contrast, the genes required for the complete oxidation of the sugar (Krebs cycle, respiration) are repressed if excess glucose is available for the bacteria. In the absence of glucose, the genes of the Krebs cycle as well as gluconeogenic genes are derepressed. The genes encoding enzymes of the pentose phosphate pathway are expressed both in the presence and the absence of glucose, as suggested by the central role of this pathway in generating anabolic precursors [8, 9].

The appearance of calium and magnesium ions in culture medium is important with regards to cell viability. Calcium ions takes part in synergistic interactions with enzymes responsible for anchoring surface proteins to the cell wall, thereby affecting the bacterium's adhesion ability [10]. Magnesium ions play a role in peptidoglycan synthesis, cell wall strength, and

the prevention of cell lysis. Previous studies on the metal binding behavior of *Bacillus subtilis* have focused on the metal binding capacity and affinity calcium and magnesium ions are both important biologically active metal ions that are some of the most abundant divalent cations in nature. We find that electrostatic effects are responsible for a strong binding between metal ions. Those could lead to the change in density, weight of *Bacillus subtilis* as well as the level of nattokinase in broth would be increased. The work has not enough data to prove the hypothesis but the data should be able to explain how the nattokinase yield and biomass production increase [7, 11].

4 CONCLUSION

Nattokinase is an essential enzyme which is added by food or functional food to dissolve blood clots. It is obtained by the *Bacillus subtilis* fermentation on glucose and soybean peptone. The maximum nattokinase enzyme yield was 69.3 ± 0.2 FU/mL in optimized medium composed of (g/L): glucose (6.10); soybean peptone (5.00), K_2HPO_4 (3.00); $MgSO_4.7H_2O$ (0.25); NaCl (4.27); $CaCl_2$ (0.05) which was higher than the initial medium by 50%.

ACKNOWLEDGEMENTS

We would like to fully appreciate the help of the teachers/professors in the Chemistry Faculty of Ho Chi Minh City University of Technology in this study. This research is funded by Technology University of Ho Chi Minh under grant number T-KTHH-2015-71.

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