IOSR Journal of Engineering (IOSRJEN) www.iosrjen.org
ISSN (e): 2250-3021, ISSN (p): 2278-8719
Vol. 04, Issue 12 (December 2014), ||V4|| PP 35-40

Optimization of the Fermentation Medium to Receive The Highest Biomass Yield By *Bacillus Subtilis* Natto And The Initial Test Of Nattokinase Yield

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ABSTRACT: A bacterium, named *Bacillus subtilis* natto, is employed in medium optimization in order to be applied for nattokinase enzyme production. Six factors including glucose (X_1), soybean peptone (X_2), K_2HPO_4 (X_3), $MgSO_4.7H_2O$ (X_4), NaCl (X_5) and $CaCl_2$ (X_6) were used to screen some effect factors in the fermentation medium for the production of biomass by Plackett – Burman design. In the range studied, soybean peptone and $CaCl_2$ had significant effect on biomass production. The optimized medium containing (g/L) glucose: 5.625, soybean peptone: 13, K_2HPO_4: 2.125, $MgSO_4.7H_2O$: 0.875, NaCl: 5, $CaCl_2$: 0.05 were used for production of biomass and the highest dried cell weight in broth was 3.033 g/L. The maximum nattokinase yield was 31.06 ± 0.297 FU/mL of substrate in optimized medium above which was higher than that in before by over 30% after 20 hours of fermentation in 37^{o}C, pH 7.5.

KEYWORDS: *Bacillus subtilis, Bacillus subtilis natto, extracellular proteases, Nattokinase, Plackett - Burman*

I. INTRODUCTION

A novel fibrinolytic enzyme from natto was introduced by Sumi (1987) and it was named nattokinase. It is one of the most considerable extracellular enzymes produced by *Bacillus subtilis* natto fermentation [1]. Besides *Bacillus subtilis*, alternative sources of obtaining this enzyme are *Pseudomonas* sp. and marine creatures. Moreover, the enzyme is also obtained from other traditional fermented foods such as Chinese douche, Korean doen-jang, Korean Chungkook-jang soy sauce. The enzyme is due to its direct fibrinolytic activity. It could be used by the oral thrombolytic therapy or via natto food [2]. As a functional food, nattokinase owns several benefits compared to the available clinical thrombolytic drugs, such as safety, low cost, confirmed efficacy, prolonged effects, preventative use, and easy oral administration [3]. Several *Bacillus* species have been found to be strongly associated with these fermented –soybean products. They can be grown over a wide pH range (with an active growth between pH 5.5 – 8.5) and produce several enzymes (for example, proteases) as well as other useful biological compounds which bring superiority for *Bacillus* species in the soybean fermentation. In this paper, the used bacterium was isolated from Vietnamese natto foods. It was identified by 16S rRNA and checked some initial tests. Because of extracellular enzymes depending on the number of spores in broth, as meaning, the level of nattokinase is high when the weight of biomass is large. That is the reason, we determine the parameter of fermentation medium by *Bacillus subtilis* natto to accumulate the large biomass in the broth [4].

Many comprehensive studies were done to determine parameters of the optimal fermentation conditions for nattokinase production. Essential nutrients are critical for enhancing nattokinase production, such as soybean peptone. Bioprocess technologies involve multiple parameters to adjust and complication so that they need effective problem solving methods. Bioprocess technologies are complicated processes which involve multiple parameters to adjust suitable condition of fermentation. An accurate mathematical model equation in order to describe the whole process, interactions among of variables, statistically designed experiments use a small set of carefully planned experiments. This method is more satisfactory and effectively than other methods such as classical one – at – a – time or mathematical methods because it can study many variables simultaneously with a low number of observations, save time and costs. The components in fermentation medium are employed to optimize batch culture for biomass production. They are designed by Plackett-Burman matrix, six factors with 12 runs. The replication per factor is enough large to analyze ANOVA. In all of these cases, a mathematical model was used to describe the relationship between biomass and the concentration of the all factors. The response surface methodology (RSM) is an effective strategy for optimizing biomass production by *Bacillus subtilis*natto in a shaken flask culture [2, 5]. The present work describes the successful optimization of a culture medium for the hyper-production of biomass by *Bacillus subtilis* natto to apply in the production of nattokinase.

II. THE MATERIALS AND METHODS

Materials, medium and culture conditions

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

The cultural medium: *Bacillus subtilis* natto grows in the nutrition broth (NB) and nutrition agar (NA), 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 and the agitation rate of 150 rpm.

Experiment designs

Checking some initial tests of *Bacillus subtilis*natto

- ✓ Observing the colonies of *Bacillus subtilis* natto.
- ✓ Observing micro-cell under microscope 100X.
- ✓ *Bacillus subtilis*natto was identified by 16S rRNA.
- ✓ Checking nattokinase activity.

Optimizing the fermentation medium for production of biomass 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 milliliter of fermentation medium.Six variables were examined in the Plackett-Burman matrix with different 12-run. We determined the dried cell weight for each validation formula and analyzed the factors that affect the biomass production by *Bacillus subtilis* natto. The main factors in experiments had p-value < 0.05. With the selected factors, we carried out the first experiments with the original values (+1, -1). After analyzing the initial experiments, we determined whether the factors having great impacts on the high regression equation suitably or not. 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 dried cell weight in broth and medium factors. The results of response optimizer that were simulated by software were measured by experiments to determine the highest actual biomass in broth. Furthermore, the level of nattokinase in broth was analyzed and compared with the initial experiments in NB medium.

The analyzing method

Analyzing biomass in fermentation broth: Broth was centrifuged at 4000 rpm for 10 minutes, removed the supernatant above, biomass was obtained and dried at 105^{0}C for 5 minutes. After that the dried cell weight was measured.

Analyzing nattokinase 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 [6].

III. RESULT AND DISCUSSION

Some initial tests of *Bacillus subtilis*natto : A bacterium that was isolated from Vietnamese natto food, is named *Bacillus subtilis* natto.

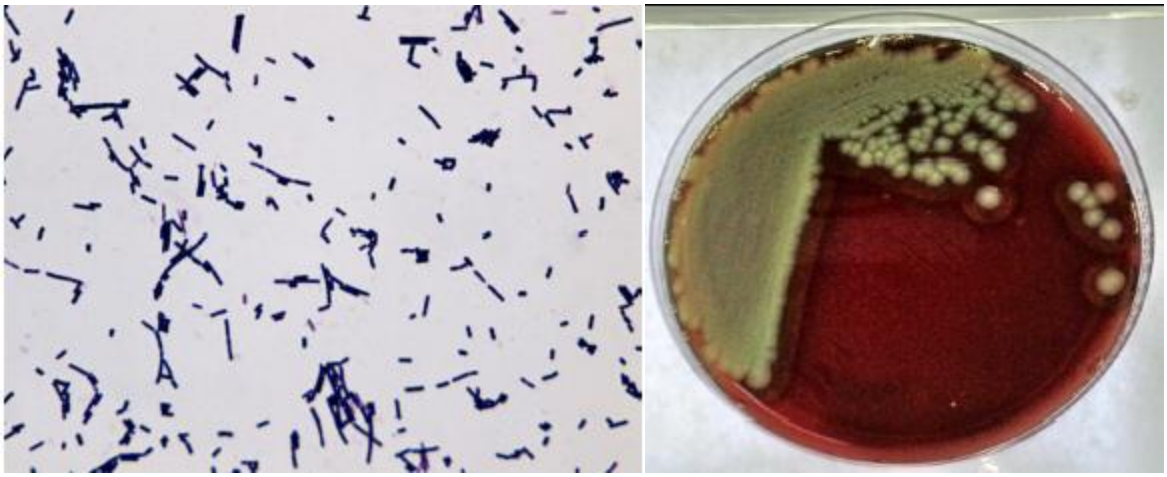

Figure 1. *Bacillus subtilis* natto under microscopes and its colony on blood agar

It is rod-shaped, oval spores, Gram-positive and the colony size is approximately 0.5- 0.8 µm x 1.5- 3.0 µm. The colonies are round, their edges are irregular jagged, their diameters are from 3 to 5 mm, and they have light yellow, dark heart. After 24- hour culture they become wrinkled surface. The bacterium that was employed to study had the 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 the16S 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. And the result of 16S rRNA was proved that the bacterium that the work was employed is named *Bacillus subtis*. Based on the result, we study the optimized fermentation medium to be obtained the maximum biomass yield.

Optimizing fermentation medium for biomass production by *Bacillus subtilis*natto

Screening main effective factors of the biomass production by *Bacillus subtilis* : The results of screening experiments were interested in the main effect factors. The efficiency of the selecting experiments and the degree of these factors' impact in the fluctuation range according to different levels were shown in Table … The obtained dried-cell weight was noticeably depended on two factors, i.e. soybean peptone (X_2) and calcium chloride (X_6) (p-value < 0.05). The polynomial regression (R_{sq} is 86.7%) is determined according to the simple function as given below:

$$\text{Response, } Y_s = 1.265 + 0.0539\ X_2 - 1.004\ X_6 \qquad (3.1)$$

In the above equation, y (g/L) symbols for dried cell weight, x_2 (g/L) symbols for added soybean peptone and x_6 (g/L) symbols for calcium chloride

Table 1. The factors in Plackett-Burman matrix and its effects on biomass production by *Bacillus subtilis*

Name of factors, g/L	**Symbols of factors**	**Values of factors**		**Main effect (R-sq = 86.7%)**	**p-value**
		Low (-1)	**High (+1)**		
Glucose	X_1	1.25	10.00	+0.164	0.266
Soybean peptone	**X_2**	**5.00**	**15.00**	**+0.539**	**0.009**
K_2HPO_4	X_3	1.25	3.00	+0.164	0.266
$MgSO_4.7H_2O$	X_4	0.25	1.50	+0.269	0.095
NaCl	X_5	2.50	7.50	-0.141	0.333
$CaCl_2$	**X_6**	**0.05**	**0.40**	**-0.351**	**0.044**

The optimal values of parameters in fermentation medium to get maximum the dried cell weight in broth

We went on conducting 9 experiments, 4 of them are (-1,1) ones and 5 are the central ones. ANOVA was carried out to statistically analyze the correlation of biomass (dried cell weight) to the two selected factors. The p-value of Lack-of-fit test was lower than 0.001 and R-sq was 90.56 %.

Table 2. Dried-cell weight in fermentation broth by RSM-CCD experiments.

RunOrder	Coded units		Uncoded units		Response, Y_CCD (dried cell weight, g/L)
	X_2	X_6	X_2 (g/L)	X_6 (g/L)	
1	-1	-1	5	0.05	2.826
2	0	0	10	0.225	2.791
3	0	0	10	0.225	2.791
4	0	0	10	0.225	2.791
5	**1**	**-1**	**15**	**0.050**	**3.072**
6	**1**	**1**	**15**	**0.400**	**3.002**
7	-1	0	5	0.225	2.580
8	**-1**	**1**	**5**	**0.400**	**2.475**
9	1	0	15	0.225	2.861
10	0	0	10	0.225	2.756
11	0	1	10	0.400	2.897
12	0	0	10	0.225	2.756
13	**0**	**-1**	**10**	**0.050**	**3.037**

This means that the arrangement of the two big effect factors (Table 2) is near the extreme of the aimed function and the polynomial regression between two selected factors and response (dried cell weight) 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 X_2 and X_6, we analyzed the 13 RMS-CCD experiments (shown in Table 2). The response that dried cell weight (g/L) in the broth ranged from 2.475 to 3.037. In the eighth-run (soybean peptone 5g/L and $CaCl_2$ 0.4 g/L), the dried cell weight was obtained lower than 2.5 g/L. Most experiments were given that the dried cell weight in the broth was higher than 3.0 g/L and those were designed with $CaCl_2$ 0.05g/L and over 5 g/L of soybean peptone.

The polynomial regression equation (R-sq = 97.08%) was shown:

$$\text{Response, } Y_{CCD}\ (g/L) = 2.643 + 0.088\ X_2 - 3.652\ X_6 - 0.004\ X_2^2 + 5.142\ X_6^2 + 0.08\ X_2.X_6 \quad (3.2)$$

In the above equation, y (g/L) symbols for dried cell weight, x_2 (g/L) symbols for added soybean peptone and x_6 (g/L) symbols for calcium chloride.

The statistical significance of the model equation was evaluated by F-test for analysis of variance (ANOVA), which showed that the regression is statistically at 99% ($p < 0.0001$) confidence level. The model F-value of 46.47 for biomass production implies that the model is statistically significant. The coefficient of determination (R^2) was calculated to be 97%, indicating that the model could explain 97% of the variability. The "lack of fit test" compares the residual error to the "Pure Error" from replicated design points. The estimated models fit the experimental data adequately. The regression equation was given that the dried cell weight in the fermentation environment was the high number of all when the concentration of soybean peptone was increased (+0.088) and the concentration of calcium chloride was decreased (-3.652). The graph below showed that the relationship between the two factors soy peptone, calcium chloride with dry biomass of the broth when hyper-production of biomass by *Bacillus subtilis* was conducted in optimal condition and medium from model 3.2.

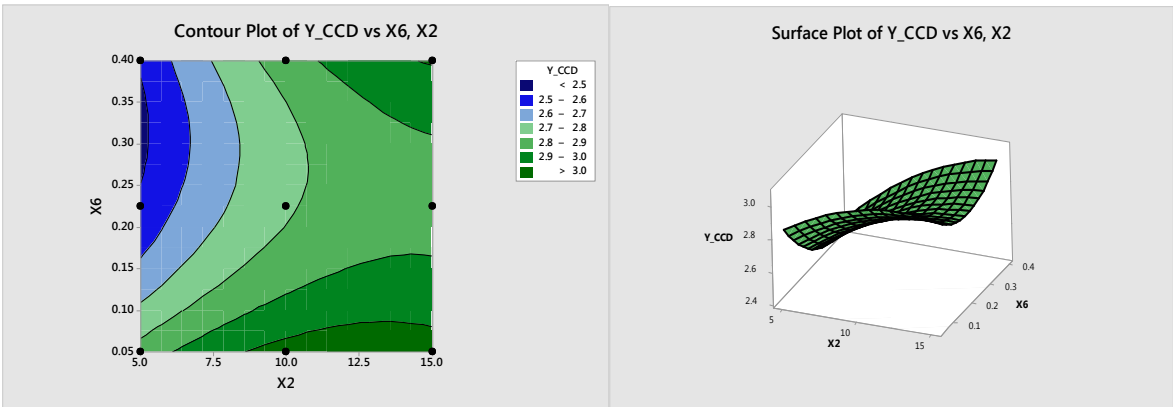

Figure 2. Contour plot and surface plot of response in RSM - CCD

From the response surface and contour plot (Fig.2) it is obvious that soybean peptone and $CaCl_2$ had a significant effect on biomass production compared to other variables. The biomass yield increased if the metal ions such as Ca^{2+} decreased, because of the enzyme activation in biochemical reactions by the metal ions.

Sumi et al. (1987) reported that the presence of a trong fibrinolytic enzyme in natto, and nattokinase may be an equally good protease for oral fibrinolytic therapy because of its confirmed safety for long-term intake, stability and the strong fibrinolytic activity. In addition, nattkinase is extracellular enzyme, the level of it depends on biomass yield. If the biomass yield is maximum, the level of nattokinase is high [1, 5].The fermented soybeans produced using bacteria, however, may have a preventive effect not only on thrombosis but also cancer. Statistical experimental design has not been widely used in the biological sciences even though it has been commonly employed in many other areas such as industrial, chemical, engineering, agricultural, medical, and food sciences. The primary reason for this is that most biological research has not been involved in many manufacturing processes. However, since genetic engineering, biomaterials, and bioprocess technologies like biodegradation and bioremediation have emerged, more people are getting interested in experimental designs to improve their biological processes and productions by shortening time and increasing efficiencies [7]. The fermentation media for biomass production was optimized using statistical method. According to the previous report using the medium components such as soybean peptone, calcium chloride resulted in maximum biomass. In addition to establishing optimal fermentation medium compositions, the present methodology also makes it possible to predict the yield if the composition of the medium is altered in some ways, by using the quadratic equation. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Thus small and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes. Therefore, with the increase in yield and productivity and simultaneous cost reduction, the industrial biomass production by *B. subtilis* can be regarded as possible and economically attractive.

Comparison biomass and the level of nattokinase between the initial experiments and experiments in optimum medium : The biomass production was 2.777 ± 0.0192 g/L in the original medium with six components at their central levels. The maximum biomass yield was 3.033 ± 0.243 g/L in optimized medium composed of (g/L): glucose (5.625); soybean peptone (13), K_2HPO_4 (2.125); $MgSO_4.7H_2O$ (0.875); NaCl (5); $CaCl_2$ (0.05) which was higher than the initial medium by 10%. Additionally, the nattokinase yield was 23.583 ± 1.539 FU/mL of substrate in NB medium. The maximum nattokinase yield was 31.06 ± 0.297 FU/mL of substrate in optimized medium above which was higher than that in before by over 30%.

The present of calcium and magnesium ions in culture medium is important in cell viability. Calcium ions take part in synergistic interactions with enzymes responsible for anchoring surface proteins to the cell wall, thereby affecting the bacterium's adhesion ability [8]. 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 the change in density and weight of *Bacillus subtilis* as well as the level of nattokinase in broth to be increased. The hypothesis cannot be proved because of lacking of data which is collected from lab-work. However these data could be able to explain how the increase of nattokinase yield and biomass production is.

IV. CONCLUSION

The maximum biomass yield was 3.033 ± 0.243 g/L in optimized medium composed of (g/L): glucose (5.625); soybean peptone (13), K_2HPO_4 (2.125); $MgSO_4.7H_2O$ (0.875); NaCl 5; $CaCl_2$ (0.05) which was higher than the initial medium by 10%. Additionally, the maximum nattokinase yield was 31.06 ± 0.297 FU/mL of substrate in optimized medium above which was higher than that in before by over 30%.

V. ACKNOWLEDGEMENTS

We would like to fully appreciate the help of my close-friend, named Tam, Tran Thi Minh in the Chemistry Faculty of Ho Chi Minh City University of Technology in this study. She helped us to design the experiments and check the papers. Moreover, we thank the Nam Khoa company for identification of the bacteria name.

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