International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 5 Issue 3 ǁ June 2016 ǁ PP.25-30

Optimization of Bacillus *Subtilis* Natto Immobilization Process on Alginate – Chitosan Complex and Its Application for Nattokinase Fermentation

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ABSTRACT: *Nattokinase is a potent fibrinolytic enzyme with the potential for fighting cardiovascular diseases. In this study, Bacillus subtilis natto were immobilized in the alginate – chitosan complex for fermentation of nattokinase enzyme. Six factors affecting the efficiency of immobilization cells were screened by Plackett – Burman design including: concentration of alginate, concentration of chitosan, pH of chitosan, concentration of $CaCl_2$, added cells density, shaking time after supplementing chitosan. Results of optimization have identified two factors affecting the efficiency of cell immobilization. They are concentration of alginate (2.5%) and added cells density (approximately 5.86 million colonies per milliliter). With these two factors optimized and others kept at the normal level, immobilization efficiency reached 90.73%. After Bacillus subtilis natto had been immobilized by optimization of parameters, we conducted application for fermenting nattokinase. For 24 hours of fermentation, nattokinase enzyme activity reached 71.80 ± 0.19 FU/ml. Immobilized Bacillus subtilis natto cells were reused 6 times and on the 6^{th} time of reuse, nattokinase enzyme activity only decreased 2.7% in compared with the 1^{st} reuse.*
Keywords: *Bacillus subtilis natto, immobilized cells, alginate, chitosan, nattokinase*

I. Introduction

Nattokinase [EC 3.4.21] is an extracellular serine protease, this enzyme was demonstrated being a fibrinolytic enzyme and considered a potential agent in the effective treatment of obstructive vascular disease [1]. This enzyme has been discovered from various food sources such as Natto of Japanese [1], Doen-jang of Korea or Dauchi of Chinese [2] and from various microbial especially the strains *Bacillus subtilis* bacteria in traditional fermented foods from soybean [3]. *Bacillus subtilis* natto strains are capable of the highest yield of nattokinase biosynthesis.

The technique to immobilize cells and enzyme by entrapment in matrix method has been applied for high efficiency in the food industrial, pharmaceutical and environmental treatment [4]. Among the carriers applied in the immobilization process, alginate and chitosan are widely used because these carriers are natural polymers and have some advantages: Entrapment within gels of natural polymer is very mild technique and damage to living cells can be minimized, easily handling, inexpensive and safe for users [5]. However, the immobilization of cells in alginate gel has the disadvantage that immobilized bacteria are surrounded by a gel network which strongly limits their movement. When growth occurs, bacteria push the gel network away and colonies containing densely packed bacteria are formed. As the colony expands, it may eventually reach the surface of the gel bead. This may lead to an eruption of the colony to the surrounding medium and limiting the reuse. Moreover, alginate gel beads are unstable in the medium containing citrate and phosphate that are often used as a buffer solution in the fermentation broth. Besides, the immobilized of cells in chitosan has some disadvantage due to the small gel pore size which limits the diffusion of nutrients and metabolic products between the fermentation broth and cells in the gel bead. In order to overcome this limitation, bacteria will be entrapped in calcium alginate gel beads, after that they are coated with chitosan. The combination of the amino group of chitosan and carbonate group of alginate will make up the gel network strength, which will help reduce cells released and increase the number of cell reuse [6,7]. In addition to that, chitosan's characteristics of antibacterial in the fermentation broth and stability at high temperatures will create favorable conditions for *Bacillus subtilis* natto cells to grow strongly [8].

II. Material And Method

1.1 Microorganism Strain And Cultural Medium

Bacillus subtilis natto was selected from bacteria collection of Ho Chi Minh University of Technology.

Bacillus subtilis natto was cultured in the nutrition broth (NB) and nutrition agar (NA), the pH 7.5, the temperature at 37^0C, the agitation of 200 rpm.

Medium for fermentation broth composed of Glucose 5.6 (g/l), Peptone 13 (g/l), $MgSO_4.7H_2O$ 0.875 (g/l), NaCl 2.5 (g/l), $CaCl_2$ 0.025 (g/l) [9].

Six factors effecting on efficiency of immobilization: concentration of alginate (1.5-3.5%), concentration of chitosan (0.2-0.6%), pH of chitosan (5-6); concentration of $CaCl_2$ (1-3%), initial supplement cells density (10 - 100 million colony forming units per milliliter); shaking time when supplementing chitosan (20-30 minutes)

1.2 Methods

2.2.1 Optimization process of Bacillus subtilis natto

- Screening the factors effecting on the efficiency immobilization of cell.

Six variables were examined in the Plackett Burman matrix with different 12 runs. We determined the performance of immobilization for each validation formula and analyzed the factors having great impacts on efficiency of immobilization cells. The main effecting factors had p value < 0.05. The chosen factors were designed to determine the relationship between those and the response.

- Optimizing parameter for cells immobilization process

With the selected factors from the screening experiments, we carried out the initial experiment with 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 are suitable or not. If the regression is 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 and determined function of the poly - nominal regression accurately to describe relationship between the efficiency of immobilization and each factors.

The optimized results of response surface methodology were calculated by software simulation to determine the highest immobilization efficiency and immobilization conditions.

Analyzing data and identifying the regression were carried out by Minitab 16.

2.2.2 Using immobilization of cells for fermentation nattokinase

We performed immobilization of cells with optimal parameters found from optimizing experiment then conducted fermenting enzyme nattokinase.

Immobilized cells were fermentation with: broth being medium for fermentation, temperature at 37^0C, agitation at 200 rpm. The survey conducted on process of enzyme nattokinase biosynthesis was based on time of fermentation by immobilization of cells. Survey process started at 8^{th} hour of fermentation and on every 4 hours, sample was taken to determine enzyme nattokinase activity. Fermentation was stopped at 28^{th} hour.

Survey reused immobilized cells by stopping fermentation at 24^{th} hour, collecting broth for determine nattokinase activity and transferring immobilized cells to fresh broth medium.

2.2.3 Analyzing nattokinase activity

Nattokinase activity was determined by ability to hydrolyze fibrin fiber. *Bacillus subtilis* natto fermentation was stopped after 20 hours and broth was centrifuged at 13000 rpm for 20 minutes and obtained the supernatant to determine nattokinase activity.

Tris-HCl (50mM, pH 7.5) of 1.3 mL and 0.4 mL 0f 0.72% (w/v) fibrinogen solution were taken in vials and kept in water bath (37^0C) for 5 minutes. Then 0.1 mL thrombin (20U/mL) was added and kept in water bath (37^0C) for 10 minutes. To this clot, 0.1 mL of enzyme was added. After incubation (37^0C, 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 of enzyme required to produce an increase in absorbance equal to 0.01 in 60 minutes at 280 nm [10].

III. Result And Discussion

1.3 Screening main effective factors of the cell immobilization efficiency

The cell immobilization efficiency and the effective values obtained from Plackett Burman matrix by experiments were illustrated in Table1. Two factors notably influenced in cell immobilization efficiency ($p<0.05$) were alginate concentration and cell density.

The alginate concentration factor and the added cells density had effect value (39.9, p = 0.01) and (45.56, 0.01) respectively. The remaining factors had not effect significantly in designed experiments.

Table 1: The factor in Plackett Burman matrix and its effect to immobilization cells

Name of factors	Symbols of factor	Values of factors		Main effect	P values
		Low (-1)	High (+1)		
Alginate concentration (%)	**X_1**	**1.5**	**3.5**	**39.9**	**0.01**
Chitosan concentration (%)	X_2	0.2	0.6	0.1	0.767
pH chitosan	X_3	5	6	0.09	0.774
$CaCl_2$ concentration (%)	X_4	1	3	5.61	0.064
The added cells density (million	**X_5**	**10**	**100**	**45.56**	**0.01**

colonies/ml)					
Shaking time	X_6	20	40	5.75	0.062

R-sq = 95.10%

1.4 The optimal values of parameters in immobilizing cell process for highest efficiency of immobilization

We determined the relationship between cell immobilization efficiency and two main effective factors through 9 initial experiments. Through initial experiments, we found that alginate concentration has greatest impact on immobilization efficiency than added cell. Through the levels of two variable and their effect on immobilization efficiency (Table 2) we have some following remarks: Specifically, alginate concentration had main effect 165.2 and P value 0.00 then added cell had main effect 31.65 and P value 0.002. The analyzing data demonstrated R-sq = 98.63% and Lack of fit test having p-value of 0.05, this meant that the immobilization efficiency and two main effective factors were not in the linear relationship.

Table 2: The affecting level of two factors

Name of factor	Symbol	Value of factor			Main effect	P value
		Low (-1)	Central (0)	High (+1)		
Alginate concentration (%)	X_1	1.5	2.5	3.5	165.02	0.000
Cell added (million colonies/ml	X_5	10	55	100	31.65	0.002

We identified the function of the poly-nominal regression accurately. The experiment for response surface methodology having the central composite designs (RMS-CCD) with the main effective factors (-α, -1, 0. +1 +α) were conducted through 13 experiments, including 5 central experiments, 4 corner experiments and 4 axial experiments.

The highest cell immobilization efficiency was the 4th run (X_1=0, X_5= -α) of all experiments, which reached 90.73%. The lowest cell immobilization efficiency was the 3rd run (X_1= -1, X_5= 1) which reached 30.23%, the efficiency of immobilization. The experiments were illustrated in Table 3.

Table 3: The efficiency of immobilization *Bacillus subtilis* natto by alginate – chitosan complex in central composite designs.

Run	Code value		Un code value		Efficiency Y (%)
	X_1	X_5	X_1 (%)	X_5 (million colony/ ml)	
1	0	0	2.5	55	75.67
2	-1	-1	1,5	10	39.76
3	**-1**	**1**	**1.5**	**100**	**30.23**
4	**0**	**-1.41**	**2.5**	**5.858**	**90.73**
5	0	0	2,5	55	74,01
6	-1.41	0	1.085	55	32,41
7	1.41	0	3.914	55	45.56
8	0	0	2.5	55	76.58
9	1	1	3.5	100	59.32
10	1	-1	3.5	10	80.27
11	0	0	2.5	55	75.85
12	0	+1.41	2.5	104.142	42.71
13	0	0	2.5	55	76.36

R-sq – 90.15%

In this case, we determined the relationship between the main effective factors and response. The poly-nominal regression equation was determined below.

$Y (\%) = -67.86 + 106.86*X_1 + 1.35*X_5 – 18.47*X_1^2 - 2.27*X_5^2 – 6.34*X_1*X_5$

In above equation, Y (%) is symbol for cell immobilization efficiency, X_1 (%) is symbol for alginate concentration, X_5 (million colonies per milliliter) is symbol for added cell density.

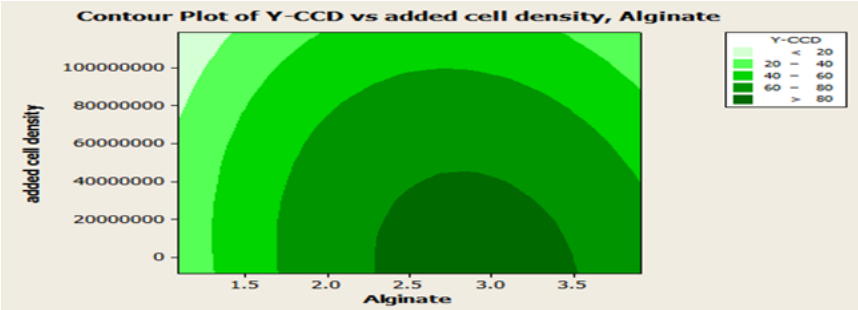

Figure 1: The equation contours show the relationship between Y (%) and X_1, X_5

When added cells density was above central value, immobilization efficiency was reduced. This shows that, added cells density being higher than central value will lead to competitive nutrients of cells in the gel bead. Besides, the increasing by-product in the fermentation medium leads to inhibition of cell grows. Therefore, cells enter death phase and the cells density in the gel bead decreases.

When alginate concentration increased above the central value, the immobilization efficiency also increased. However, when alginate concentration reached value +1, despite the increasing in alginate of concentration the immobilization efficiency was not increased.

The reason is that when alginate concentration increases, the viscosity increases too, so the ability of diffusing cells into alginate suspensions is limited. On the other hand, when alginate concentration decreases below the central value, the immobilization efficiency also decreases because low alginate concentration lead to the large size pore gel, cells in gel bead are easily released to fermentation medium.

The maximum efficiency of immobilization Bacillus subtilis natto on alginate – chitosan complex was 90.73% when alginate concentration is 2.5% and added cell density is 8.58 million colonies per milliliter, the others factors remaining at central level.

1.5 Using immobilization Bacillus subtilis natto for fermentation of nattokinase

1.5.1 Investigating biosynthesis process of nattokinase enzyme based on time of fermentation.

Survey time period started at 8th hour and stopped at 28th hour of fermentation. The nattokinase enzyme activity obtained increased as survey time increased. At the time of 8th hour after fermentation, nattokinase enzyme activity reached 31.46 FU/ml because in this stage, cells was in log phase and using nutrition for growing. The results of survey process were illustrated in the figure 2.

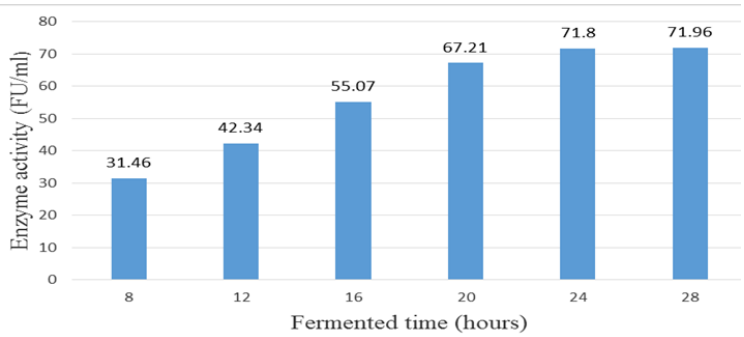

Figure 2: The effect of fermenting time on biosynthesis of nattokinase enzyme process.

After 20 hours of fermentation, nattokinase enzyme activity reached 67.21 FU/ml and increased 2.2 fold compared with at the 8th hour of fermentation. The time from 24th hour to 28th hour correspond with the time when cells in the stationary phase so nattokinase enzyme activity just slightly increased by 0.16 FU/ml. This indicated that the increase in density of cells in the fermenting medium is proportional with obtained nattokinase enzyme activity. At 28th hour of fermentation nattokinase enzyme activity reached maximum 71.96 FU/ml. However for the 4 hours prolonged time, nattokinase enzyme activity only increased 0.16 FU/ml having no economic effect. Because the purpose of fermenting nattokinase enzyme by immobilized cells is to obtain the highest enzyme activity and cell density, we chose the time to stop fermentation at the 24th hour.

1.5.2 Comparing nattokinase enzyme activity obtained from fermenting process using free cells and fermenting process using immobilized cells.

From the investigating results obtained from biosynthesis of nattokinase enzyme process based on fermentation time, we conducted fermenting immobilized cells and fermenting free cells with the same conditions. The result was illustrated in figure 3.

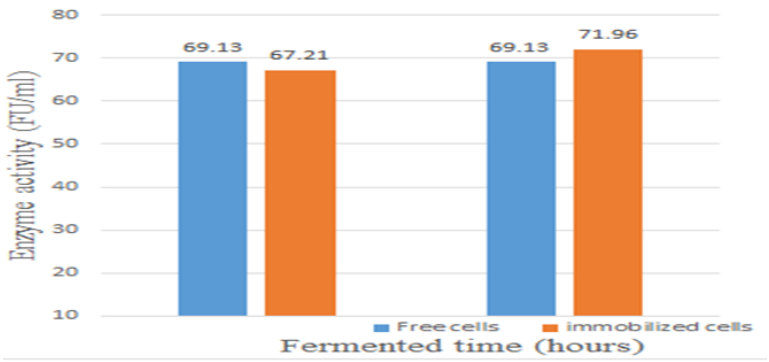

Figure 3: Comparing nattokinase enzyme activity value obtaining from fermenting free cells and fermenting immobilized cells.

For the free cells experiment, the maximum nattokinase enzyme activity obtained at 20th hour of fermentation was 69.13 FU/ml. Meanwhile, the maximum nattokinase enzyme activity obtained from fermenting the immobilized cells was 71.96 FU/ml at 28th hour of fermentation. The cause of the difference is that immobilized cells take longer time to adapt with fermentation medium than free cells do. With free cell, when cells were added in to fermentation broth, they immediately and directly contacted with the substrate. As for the immobilized cells, cells were locked up behind the barrier layer of particle gel so cells used substrate after being diffused through the membrane. On the other hand, the diffusion also has the limitation because it is effected by concentration of substrate and size of the substrate. However for free cell, when increase time for fermented from 20th hour to 28th hour, the nattokinase enzyme activity obtained was not increase. While the nattokinase enzyme activity obtained by the immobilized cell was increased 4.75 FU/ml.

1.5.3 Investigating nattokinase enzyme activity in a series of immobilized cells reuse

We conducted survey nattokinase enzyme activity for a number of immobilized cells reuse times. In every 24 hours, we stopped the fermentation, transferred gel beads to fresh broth and continued fermenting process with the same condition on the fresh broth. The results of investigating nattokinase enzyme activity over time of reuse were illustrated in the figure 4.

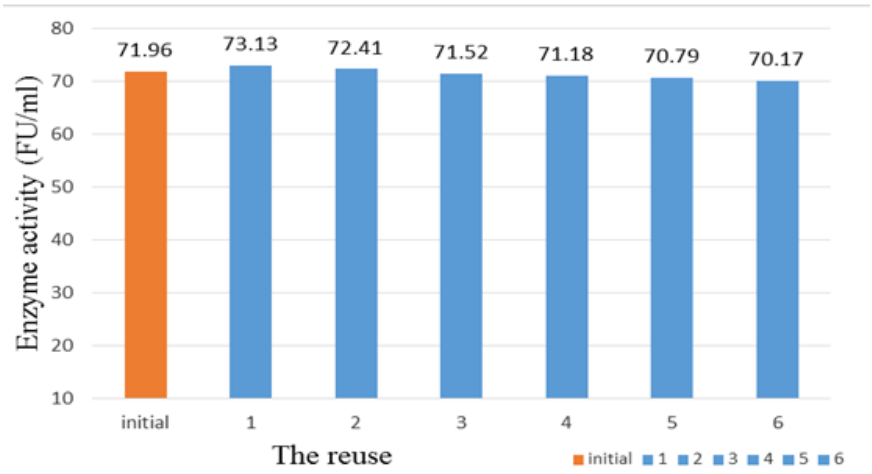

Figure 4: Survey reusing the immobilized cells

The nattokinase enzyme activity in the first reuse reached 73.13 FU/ml, the highest level among the reuse times and higher than the initial of immobilized cells. Particularly, the nattokinase enzyme activity obtained in the first reuse and the second reuse were higher than the initial of immobilized cells by 1.33 FU/ml and 0.61 FU/ml respectively. The cause of this difference is due to when we stopped process of fermented at the time of 24th hour, the cells in gel beads are ungrowing hormogenous. Some are growing, others might be in the process of biosynthesis of nattokinase enzyme thus nattokinase enzyme activity obtained was not in the highest level at the initial fermentation of the immobilized cells. At the time of the first reuse nattokinase enzyme activity obtained

reached the highest level because cells in the gel bead are not undergo growth stages which mainly using nutrition for the biosynthesis of nattokinase enzyme process. At the next time reuses, obtained nattokinase enzyme activity decreasing compared with the previous reuses may be due to after each reuse, a number of cells were being released in to fermentation broth. So cells density in the gel beads were decreased and obtained nattokinase enzyme activity were also decreased.

However after reusing for 6 times, obtained nattokinase enzyme activity compared with the 1st time of reuse is decrease by 2.73 FU/ml, this reduction is not significant. The survey result suggests that alginate – chitosan complex had its effect in reducing cells release and increasing times of reuse.

IV. Conclusion

By the response surface methodology, we had investigated two factors affecting the immobilization efficiency are: alginate concentration and added cell density. The Optimized of parameters for six factors are concentration of alginate (2.5%), concentration of chitosan (0.4%), pH of chitosan (5.5), concentration of $CaCl_2$ (2%), added cell density (approximately 8.58 colonies per milliliter) and shaking time when supplement chitosan (30 minutes). The highest of immobilization efficiency reached 90.73%.

The immobilized cells were reused 6 times and obtained nattokinase enzyme activity in the 1st time of reuse and in the 6thof reuse are 73.13 ± 0.14 FU/ml and 70.40 ± 0.09 FU/ml respectively.

Acknowledgment

We would like to full appreciate the help of the professors/teachers in the Chemistry Faculty of Ho Chi Minh University of technology in this study. This review is funded by Vietnam National University Ho Ch iMinh City (VNU-HCM) under grant number C2016-20-31.

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